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13. ABSTRACT (Maximum 200 Words)

Several oncogenes, growth factors, hormones and hypoxia have been shown to up-regulate Vascular Endothelial Growth Factor (VEGF), an essential angiogenic factor for the progression of breast carcinomas. We previously demonstrated that he angiogenic factor CCN1 (CYR61), a member of the CCN family of growth regulators, is differentially expressed in breast cancer cells overexpressiong Heregulin (HRG), a member of the epidermal growth factor-like growth factor family that regulates angiogenesis via up-regulation of VEGF expression and secretion. More recently, we found that CCN1-induced tumors in ovariectomized athymic nude mice did resemble human invasive carcinomas with increased vascularization and overexpression of VEGF. CCN1 may stimulate tumor vascularization by acting as an angiogenic inducer of endothelial cells, as VEGF does. However, little is known the regulatory role of CCN1 on the secretion of VEGF in breast epithelial cancer cells. Here, we demonstrate that HRG-stimulated secretion of VEGF in human breast cancer cells requires an atuocrine action of HRG on HER-2/neu-dependent signaling. In this regard, we generated a deletion mutant of HRG (HRG-M4) lacking the N-terminus sequence and the cytoplasmic-transmembrane region of HRG protein, which did not stimulate either HER-2/neu phosphorylation of VEGF secretion. Interestingly, we provide the first evidence that CCN1 stimulates VEGF secretion independently of HRG overexpression and/or HER-2/neu activation. Moreover, we reveal that CCN1 synergistically enhances HRG-stimulated secretion of VEGF via activation of MAPK and PI-3'K/AKT signaling pathways. In addition, we show that the involvement of CCN1 in $VEGF_{165}$ secretion can be attributed, at least in part, to its interaction with its integrin receptor $\alpha v\beta 3$ in human breast cancer cells. From a clinical perspective, current and future antagonists of specific integrins, such those used in this study directed against $\alpha v\beta 3$, or more specific anti-HRG and anti-CCN1 strategies, may have the potential to suppress tumorigenicity and metastasis of HRG- and CCN1-overexpressiong breast carcinomas by decreasing VEGF-dependent breast cancer angiogenesis.

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INTRODUCTION

Accumulating evidence supports an association between angiogenesis and the processes of tumor invasion and metastasis. In this way, several angiogenic factors and their receptors have been identified as important mediators of angiogenesis (1). We as well others have accumulated evidence suggesting that **HEREGULIN** (HRG), a growth factor involved in the progression of breast cancer to a more malignant phenotype (2-4), also is a potent stimulator of *in vitro* growth of a special blood vessel cells found in the umbilical cord, called HUVEC (Human Umbilical Vein Endothelial Cells). Hence, HRG pathway can enhance tumor neovascularization and this up-regulation of angiogenesis may contribute to a more aggressive disease. Significantly, we have also demonstrated that the growth factor-inducible immediate-early gene **CCN1** (Cysteine-rich angiogenic protein 61; **CYR61**) is a down-stream effector of HRG-induced breast cancer chemomigration and metastasis, probably through interactions with the **$\alpha_v\beta_3$ integrin receptor** (5). CCN1 stimulates chemotaxis in endothelial cells and induce neovascularization *in vivo* (6). Moreover, we have shown that CCN1 overexpression in tumor cells enhances tumorigenicity by increasing tumor size and vascularization (7). In this regard, we have determined previously that CCN1 gene expression is elevated in highly invasive and metastatic human breast cancer cells and tumor biopsies (5). Accordingly, CCN1 overexpression is correlated with more advance stage of malignancy in patients samples (7). Taken together, these findings prompted us to hypothesize that HRG (directly or indirectly through CCN1) is an important regulator of the vascular compartment in breast cancer with stimulating effects on tumor neovascularization which, in turn, promotes progression and dissemination of breast carcinoma.

We recently showed that in ovariectomized nude mice brast carcinoma cells secreting HRG promoted more vascularized tumors (9). We demonstrated that one of the mechanisms by which HRG achieved this aggressive phenotype was mediated *via* an increase in the expression of Vascular Endothelial Growth Factor (VEGF), a key tumor angiogenic factor. In MCF-7/HRG-derived tumors, a great increase in VEGF expression was observed by inmunohistochemistry staining with anti-VEGF antibody. These results were further confirmed by an ELISA assay, demonstrating a 3- to 8-fold increase in VEGF expression was observed in the conditioned media from HRG-transfected cells. Of interest, in our experiments there was a positive correlation between the increase in the ability of the HRG transfectants to secrete VEGF and the levels of HRG expression. Consistent with this finding, HRG has been shown to selectively up-regulate VEGF secretion in both cancer and HUVEC cells and to stimulate *in vivo* angiogenesis (10). Nevertheless, some of the effects that were observed *in vivo* were probably mediated indirectly *via* the up-regulation of other genes in an autocrine/paracrine manner. For example, the expression of CCN1 was significantly up-regulated in the MCF-7/HRG-derived tumors. Accordingly, we found that CCN1-induced tumors in ovariectomized athymic nude mice did resemble human invasive carcinomas with increased vascularization and overexpression of VEGF (7).

An important issue that arises from the contribution of VEGF to breast cancer neovascularization is an understanding of the mechanism(s) that regulate VEGF expression. Such mechanisms are important not only for VEGF signaling in breast cancer cells, but also for angiogenesis as well. Clearly, hypoxia is a strong inducer of VEGF transcription and mRNA stability (11), but other factors are likely to be involved. Of note, our finding that the $\alpha_v\beta_3$ integrin can promote the survival of breast carcinoma cells in stress conditions such as chemotherapy treatment is intriguing (12), and raised the novel possibility that a specific integrin $\alpha_v\beta_3$, which has been implicated in breast cancer progression (13), could play an active role regulating VEGF secretion in HRG-overexpressing breast cancer cells.

BODY

The ***aim 2*** in the proposed study was to determine the effect of HRG on the secretion of angiogenic factors and elucidate the contribution of HRG to the angiogenic potential of breast carcinomas. Since VEGF appears to be an essential angiogenic factor for the progression of many solid tumors, including breast carcinomas, we examined the role of HRG on the regulation of the VEGF secretory isoform, $VEGF_{165}$, in relation to the expression level of either CCN1 (CYR61) or *HER-2/neu* oncogene in human breast cancer cells.

Several oncogenes (*HER-2/neu*), growth factors (HRG), hormones and hypoxia have been shown to upregulate $VEGF_{165}$. We previously demonstrated that CCN1 is differentially expressed in breast cancer cells overexpressing

HRG, a member of the epidermal growth factor-like growth factor family that regulates angiogenesis *via* up-regulation of $VEGF_{165}$ expression and secretion (5, 10). More recently, we found that CCN1-induced tumors in ovariectomized athymic nude mice did resemble human invasive carcinomas with increased vascularization and overexpression of $VEGF_{165}$ (7). CCN1 may stimulate tumor vascularization by acting as an angiogenic inducer of endothelial cells, as $VEGF_{165}$ does (14). In addition, CCN1 may act as a chemotactic, mitogenic, and matrix-remodeling factor as previously demonstrated in fibroblasts (12, 15-17). However, little is know the regulatory role of CCN1 on the secretion of $VEGF_{165}$, an angiogenic factor of reference, in human breast cancer cells.

In this annual report, we demonstrate that HRG-stimulated secretion of $VEGF_{165}$ in human breast cancer cells requires an autocrine action of HRG on *HER-2/neu*-dependent signaling. In this regard, we generated a deletion mutant of HRG (HRG-M4) lacking the N-terminus sequence and the cytoplasmic-transmembrane region of HRG protein, which did not stimulate either *HER-2/neu* phosphorylation or $VEGF_{165}$ secretion. Interestingly, we provide the first evidence that CCN1 stimulates $VEGF_{165}$ secretion independently of HRG overexpression and/or *HER-2/neu* activation. Moreover, we reveal that CCN1 synergistically enhances HRG-stimulated secretion of VEGF *via* activation of MAPK and phosphatidylinositol 3'-kinase (PI-3'K)/protein kinase B (AKT) signaling pathways. In addition, we show that the involvement of CCN1 in $VEGF_{165}$ secretion can be attributed, at least in part, to its interaction with its integrin receptor $\alpha_v\beta_3$ in human breast cancer cells.

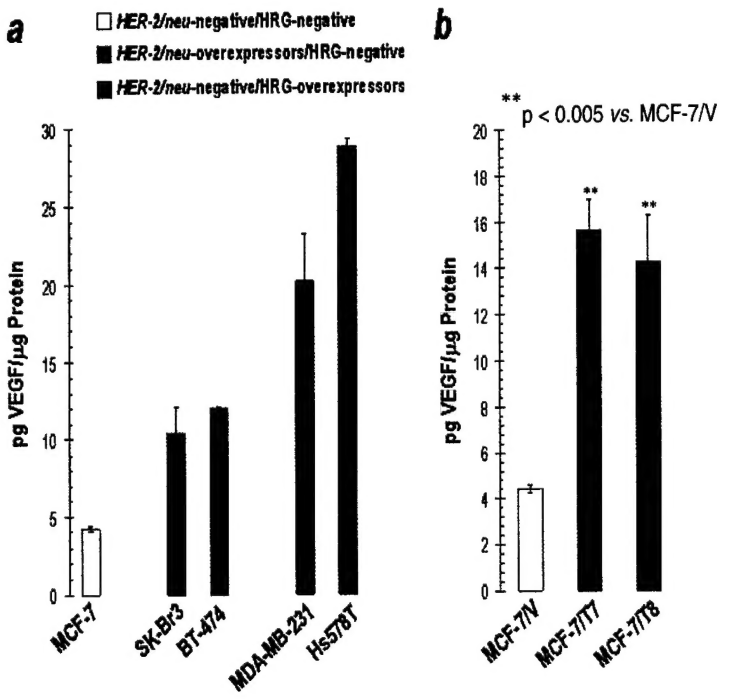


Figure 1. $VEGF_{165}$ secretion levels in human breast cancer cells overexpressing either *HER-2/neu* or HRG.

1. High levels of $VEGF_{165}$ secretion correlate with overexpression of HRG and CCN1 in human breast cancer cells. Figure 1a shows the basal level of the VEGF secretory isoform, $VEGF_{165}$, in human breast cancer cell lines naturally overexpressing either *HER-2/neu* (SK-Br3 and BT-474) or HRG and CCN1 (MDA-MB-231, Hs578T) as compared to $VEGF_{165}$ secretion levels in MCF-7 breast cancer cells, which express physiological levels of *HER-2/neu* and HRG. Cells were seeded at a concentration of 5 x 10^5 using 100-mm plates, and kept undisturbed until 75% confluence. Next, cells were starved for 24 h in serum-free medium, and maintained in 0.5% FBS for 48 h. Conditioned media were then collected and $VEGF_{165}$ expression was determined using the Quantakine human VEGF Immunoassay kit (R&D Systems, Minneapolis, MN, USA). VEGF values were normalized to total protein concentration in each plate. For calibration, we used human recombinant $VEGF_{165}$ provided by the supplier to construct a standard curve and to obtain absolute values of VEGF protein content. HRG-overexpressing MDA-MB-231 and Hs578T breast cancer cell lines demonstrated VEGF secretion levels (20.2 ± 3 and 28.8 ± 0.5 pg VEGF/µg protein, respectively) beyond the up-regulation mediated by overexpression of *HER-2/neu* in SK-Br3 and BT-474 breast cancer cell lines (10.4 ± 1.8 and 11.9 ± 0.1 pg VEGF/µg protein, respectively), when compared with low HRG- and *HER-2/neu*-expressing MCF-7 cells (4.2 ± 0.2 pg VEGF/µg protein). Next, we looked at MCF-7 cells that express low levels of *HER-2/neu*, and the same cells engineered to overexpress HRG (Figure 1b). The basal level of VEGF secretion was significantly increased in MCF-7/HRG transfectants (up to 15.7 ± 1.3 pg VEGF/µg protein in the MCF-7/T7 clone). This observation was not a clonal selection effect since a significant induction of $VEGF_{165}$ secretion was found in MCF-7

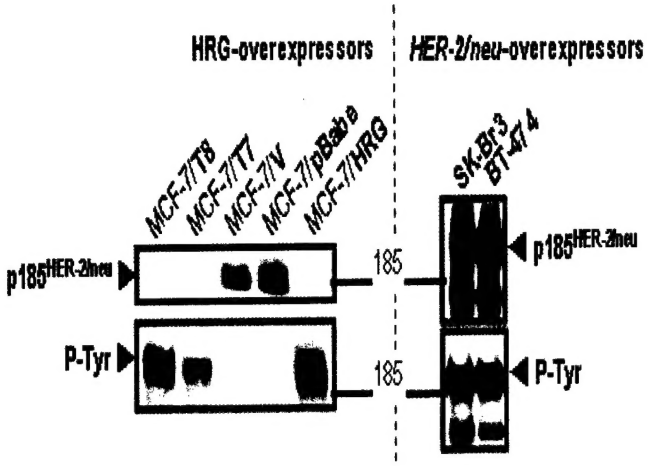
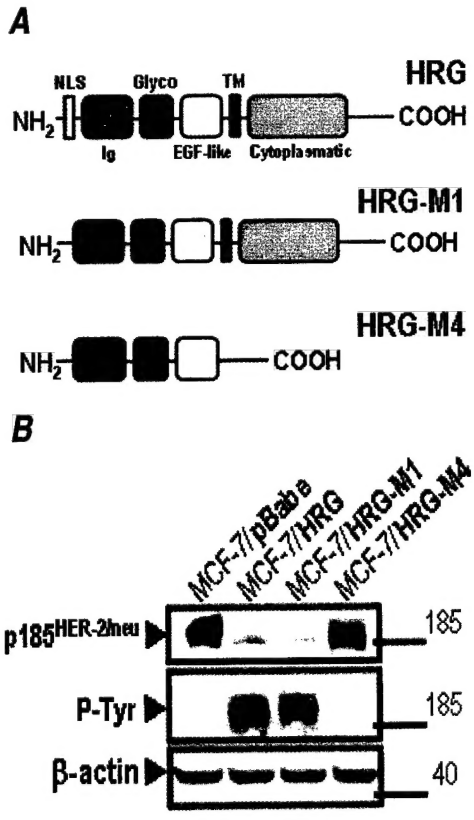


Figure 2. *HER-2/neu* protein expression is reduced by HRG overexpression. MCF-7/V, MCF-7/pBabe, MCF-7/T7, MCF-7/T8, MCF-7/HRG, SK-Br3, and BT-474 were grown in IMEM/5% FBS until reaching 75% confluence. Cells were then washed with PBS and solubilized in lysis buffer containing phosphatase and protease inhibitors. 50 μg of protein per sample was subjected to Western blotting with $p185^{HER\text{-}2/neu}$ or P-Tyr antibodies.

cells stably transduced with a pBabe retroviral vector containing the identical HRG cDNA (MCF-7/HRG cells). We examined by Western blotting the effect of forced expression of HRG on the expression of *HER-2/neu* protein in MCF-7 breast cancer cells. The densitometric analysis suggested that *HER-2/neu* protein expression was up to 80% lower in the HRG-transfected MCF-7 cells (MCF-7/T7 and MCF-7/T8 clones) or pBabe-HRG-transduced MCF-7 cells (MCF-7/HRG) than in matched control cells (MCF-7/V and MCF-7/pBabe, respectively) or in wild-type MCF-7 cells (Figure 2). HRG overexpression resulted in a constitutive activation of *HER-2/neu* receptor, as evidenced by the $p185^{HER\text{-}2/neu}$ tyrosine phosphorylation levels observed in T7, T8, and MCF-7/HRG cells. The differences in $p185^{HER\text{-}2/neu}$ phosphorylation between MCF-7/T clones reflected a variation of HRG expression between different clones (4). Tyr-phosphorylated $p185^{HER\text{-}2/neu}$ was undetectable in MCF-7/V, MCF-7/pBabe, and wild-type MCF-7 cells. Importantly, $p185^{HER\text{-}2/neu}$ phosphorylation levels in MCF-7 cells engineered to overexpress HRG were comparable to those found in SK-Br3 and BT-474 cells, which are known to overexpress *HER-2/neu*. These results indicate that high levels of HRG, independently of *HER-2/neu* overexpression, leads to up-regulation of $VEGF_{165}$ secretion in human breast cancer cells.

2. HRG-stimulated secretion of $VEGF_{165}$ requires an autocrine action of HRG on *HER-2/neu*-dependent signaling. HRG-induced responses are mainly mediated by the *HER* family of tyrosine kinase receptors (*HER-2/-3/-4*). MCF-7/T clones and MCF-7/HRG cells produce high levels of HRG and thus have constitutively activated *HER-2/neu* receptors in spite of their low *HER-2/neu* receptors expression (Figure 2). To investigate whether up-regulation of $VEGF_{165}$ secretion plays a role in HRG-promoted aggressive phenotype of breast cancer cells, we evaluated $VEGF_{165}$ levels in MCF-7 cells engineered to express a deletion mutant of HRG (HRG-4) incapable of promoting tumorigenicity (18). HRG-M4 is a structural mutant of HRG β-2 that lacks N-terminus sequences (a putative nuclear localization signal -NLS-) and the cytoplasmic domain of the protein (Figure 3*A*). We previously demonstrated that HRG-4 protein, although stably expressed in MCF-7 cells, is sequestered into a cellular compartment and is not secreted into the culture media, thus preventing its autocrine action and *HER-2/neu* autophosphorylation. In addition, MCF-7/HRG-M4 cells did not become more aggressive or estrogen-independent, which was opposed to the phenotype arising from the full-length HRG protein (18). Here, MCF-7 cells were transduced with the deletion mutant of HRG (MCF-7/HRG-M4 cells) or with the empty retroviral vector (MCF-7/pBabe) to circumvent the possibility of



clone variations, and a stable MCF-7/HRG-M4 cell line was expanded after selection in puromycin containing media. As expected, Western blotting analysis of MCF-7/HRG-M4 cells demonstrated neither down-regulation of p185$^{HER\text{-}2/neu}$ receptor nor increase in p185$^{HER\text{-}2/neu}$ tyrosine phosphorylation when compared with the matched control cells (Figure 3*B*). Interestingly, the transduction of MCF-7 cells with the HRG deletion mutant M4 did not cause any increase in $VEGF_{165}$ secretion when compared to the empty-vector infected MCF-7 cells (Figure 3*C*). This result indicates that deletion of the NLS sequence and the cytoplasmic domain of the full-length HRG protein abolish its ability to up-regulate $VEGF_{165}$ secretion in MCF-7 breast cancer cells. Moreover, HRG-induced over-secretion of $VEGF_{165}$ is likely dependent from activation of the *HER-2/neu*-dependent signaling.

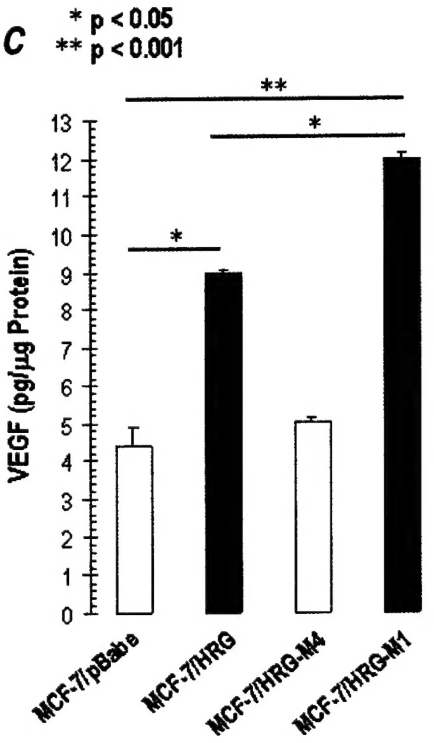


Figure 3. Role of HRG structural domains in HRG-stimulated $VEGF_{165}$ secretion. ***A.*** Schematic representation of HRG-deletion mutants M1 and M4. *NLS*: Nuclear localization signal; *Ig*: Immunoglobulin like domain; *Glyco*: Glycosylation domain; *EGF-like*: EGF-like domain. *HRG*: Wild-type protein. *HRG-M1*: NLS-deletion mutant of HRG. *HRG-M4*: NLS- and COOH-deletion mutant of HRG. This mutant is lacking the NLS and cytoplasmic domains of HRG. ***B.*** *Top*, MCF-7/pBabe, MCF-7/HRG, MCF-7/HRG-M1, and MCF-7/HRG-M4 cells were grown in IMEM/5% FBS until reaching 75% confluence. Cells were then washed with PBS and solubilized in lysis buffer containing phosphatase and protease inhibitors. 50 µg of protein per sample was subjected to Western blotting with p185$^{HER\text{-}2/neu}$ or P-Tyr antibodies. ***C.*** $VEGF_{165}$ secretion levels in MCF-7 human breast cancer cells engineered to overexpress either full-length HRG (MCF-7/HRG) or deletion mutants of HRG (HRG-M4 and HRG-M1).

2. HRG-stimulated secretion of $VEGF_{165}$ does not require a nuclear localization of HRG. HRG secretion to the extracellular media activates the *HER* receptors. Interestingly, HRG also localizes in the nuclei as previously observed in cells treated with ^{125}-I-HRG protein or transfected with the HRG cDNA (18, 19). It is not clear, however, which functions can be attributed to the nuclear HRG and which functions, if any, can be independent of *HER* receptor activation. Therefore, we investigated whether secretion of HRG followed by activation of *HER-2/neu* receptor are *necessary* and/or *sufficient* molecular events in the HRG-promoted secretion of $VEGF_{165}$. Our previous studies, using GFP-tagged HRG demonstrated a clear nuclear localization of the extracellular domain of the protein (18). Interestingly, this localization was independent of the localization of the *HER* receptors, which have been previously identified in the nucleus of mammary epithelial cells, as assessed by co-localization experiments using confocal microscopy (data not shown). Therefore, we concluded that HRG must have a NLS at the NH_2-terminus. Likewise, we identified a novel NLS in the extracellular domain of the HRG-protein between the fourth and the sixteenth amino acids, which does not fully resemble any of the known nuclear localization sequences, but has close homology to the NLS that is found in the p53 protein. In order to confirm its functionality, we deleted the first 33 amino acids of the HRG sequence, containing the putative NLS, and replaced it with GFP (NLS-) HRG. The HRG-negative, mammary epithelial breast cancer cell line MCF-7, was transfected with the (NLS-) HRG or full–length HRG-GFP fusion expression plasmids, and the localization of the fusion proteins was visualized by confocal microscopy. We observed a perinuclear localization of the HRG protein lacking the NLS sequence, which was markedly different from the clearly nuclear localization of the full-length HRG protein (data not shown). These results confirm that HRG contains a functional NLS, which is essential for the translocation of the growth factor to the nucleus in MCF-7 cells. Based on these studies we set to identify if the nuclear localization of HRG was required for the up-regulation of $VEGF_{165}$ secretion. We found that the expression of the HRG-M1 increased the expression of $VEGF_{165}$ to even higher levels then the full-length HRG (Figure 3*C*).

We investigated whether the phenotypic changes that are mediated by deletion of the NLS of HRG are mediated through changes in the levels of *HER-2/neu* phosphorylation. Conversely to HRG-M4, as HRG-M1 protein is secreted, MCF-7/HRG-M1 cells did behave similarly to the MCF-7 cells infected with the wild-type HRG, and demonstrated down-regulation of *HER-2/neu* protein expression and constitutive activation of $p185^{HER\text{-}2/neu}$ (Figure 3*B*). These results indicate that the nuclear NH_2-terminus of HRG acts as a suppressor rather than an activator of $VEGF_{165}$ secretion in human breast cancer cells.

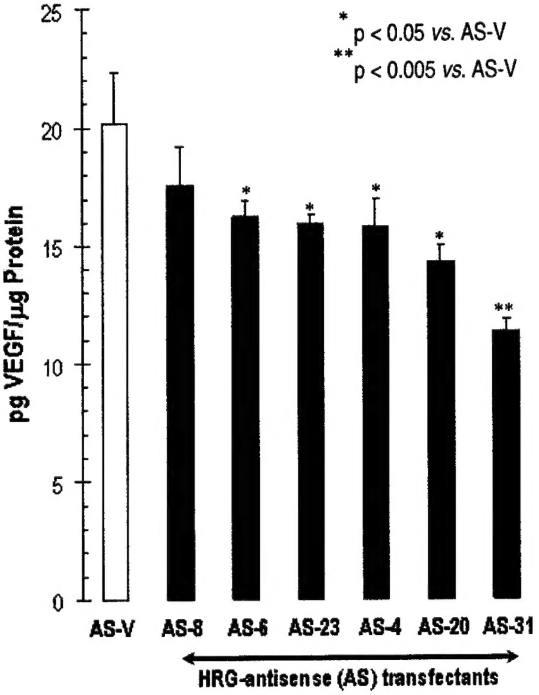


Figure 4. $VEGF_{165}$ secretion levels in antisense-HRG MDA-MB-231 human breast cancer cells.

3. Blockade of HRG expression down-regulates secretion of $VEGF_{165}$ in MDA-MB-231 breast cancer cells. HRG-overexpressing MDA-MB-231 cells express low to undetectable levels of *HER-2/neu*. Therefore, the receptor signaling events that lead to over-secretion of $VEGF_{165}$ in HRG-overexpressing MDA-MB-231 cells are not dependent upon *HER-2/neu* overexpression, but rather on ligand (HRG)-induced receptor heterodimerization. To further support this hypothesis, we evaluated the levels of $VEGF_{165}$ secretion on MDA-MB-231 cells in which HRG expression was diminished by transfection with the HRG-β2 cDNA (amino acids) oriented from 3' to 5' end, that is, in an antisense direction (20). Conditioned media from six HRG antisense (HRG-AS) clones were analyzed for $VEGF_{165}$ secretion levels and compared to those found in empty vector-transfected MDA-MB-231 (MDA-MB-231/AS-V) cells. Secretion of $VEGF_{165}$ was significantly reduced in the majority of HRG-AS clones. In fact, $VEGF_{165}$ secretion was reduced by about 50% in MDA-MB-231/AS-31 transfectants (from 20.2 ± 2 in AS-V to 11.4 ± 0.5 pg VEGF/µg protein) which express low to undetectable levels of HRG (20). These results demonstrate that the decreased levels of HRG expression in the HRG-AS transfectants correlate with their decreased ability to secrete $VEGF_{165}$.

4. Overexpression of CCN1 (CYR61) Stimulates Secretion of $VEGF_{165}$ in MCF-7 Breast Cancer Cells. We have previously demonstrated that CCN1 is highly and selectively expressed in human breast cancer cells that naturally overexpress HRG (5, 12). Accordingly, CCN1 overexpression was also found in MCF-7 human breast cancer cells engineered to overexpress HRG (5). To determine whether overexpression of CCN1 was *sufficient* to stimulate secretion of $VEGF_{165}$ in the absence of HRG- and *HER-2/neu* overexpression, CCN1 was introduced in MCF-7 breast cancer cells, which are HRG-negative, and express physiological levels of *HER-2/neu* (7). CCN1-overexpressing MCF-7/C2-6, MCF-7/C2-9, and MCF-7/C2-2 transfectants demonstrated $VEGF_{165}$ secretion levels (6.3 ± 0.7, 6.7 ± 0.1, and 10.2 ± 0.1 pg VEGF/µg protein, respectively) significantly higher than those found empty vector-transfected MCF-7 cells (4.2 ± 0.8 pg VEGF/µg protein). We

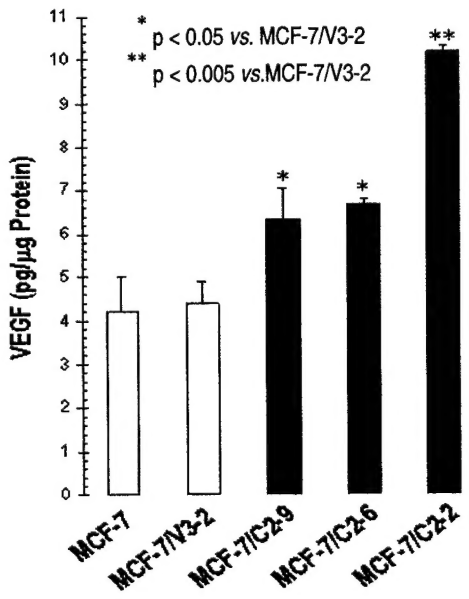


Figure 5. $VEGF_{165}$ secretion levels in MCF-7 cells engineered to overexpress CCN1.

also assessed the expression and activation status of *HER-2/neu* in the control empty-vector and the MCF-7 clones stably expressing CCN1. As expected, no significant changes in either the expression or tyrosine phosphorylation of $p185^{HER\text{-}2/neu}$ was detected in MCF-7/CCN1 clones (data not shown). Taken together, these *in vitro* findings suggest that CCN1 overexpression is *sufficient* to up-regulate $VEGF_{165}$ secretion in the absence of HRG-induced transactivation of *HER-2/neu* signaling.

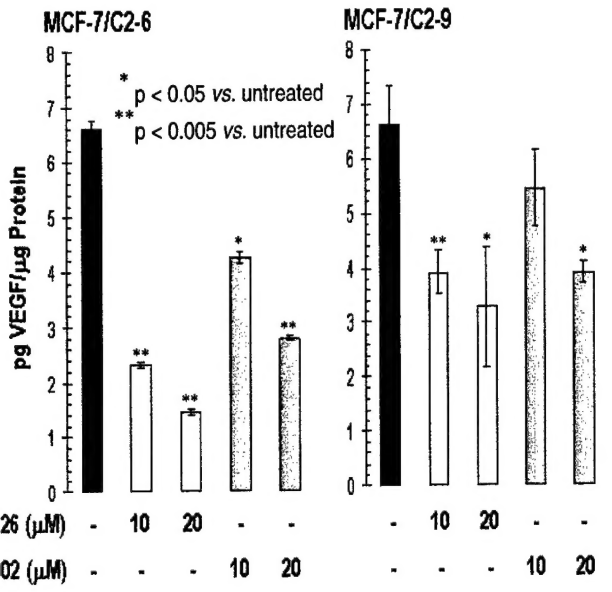


Figure 6. Role of MAPK and PI-3'K/AKT signaling pathways in CCN1-induced up-regulation of $VEGF_{165}$ secretion.

5. Overexpression of CCN1 (CYR61) Stimulates Secretion of $VEGF_{165}$ *via* Activation of MAPK and PI-3'K/AKT cascades in MCF-7 breast cancer cells. Several *HER*-coupled signaling pathways including the MAPK pathway mediate VEGF by hypoxia (21). Furthermore, inhibition of PI-3'K, which has been shown to play an important role in *erb*B signaling, particularly with respect to *HER*-3/*HER*-2 heterodimers, leads to inhibition of VEGF expression and angiogenesis (22, 23). Therefore, we next tested the contribution of each particular pathway on CCN1-stimulated secretion $VEGF_{165}$ in MCF-7 breast cancer cells. We first examined the total levels of MAPK and AKT and their phosphorylation status, *i.e.* activation, in the same protein lysates from CCN1-overexpressing MCF-7 cells in which $VEGF_{165}$ secretion levels were found to be up-regulated. Significantly higher levels of activated MAPK were observed MCF-7/C2-2, MCF-7/C2-6, and MCF-7/C2-9 clones but not in control cells, whereas the total level of MAPK was similar in CCN1-overexpressing and matched control cells (data not shown). Similarly, CCN1-overexpressing MCF-7/C2-2, MCF-7/C2-6, and MCF-7/C2-9 transfectants demonstrated an up-regulation of active AKT (as measured by antibodies specific for phosphor-Ser473 AKT) compared with matched control cells, whereas the level of total AKT were unchanged. The constitutive levels of phosphorylated AKT in MCF-7/CCN1 clones were at least 5-fold higher than in the parental MCF-7 cells, when the levels of phosphorylation were normalized to total AKT protein that was loaded (data not shown). These data demonstrate that stimulation of VEGF secretion in CCN1-overexpressing MCF-7 cells coincides with increased activation of MAPK and PI-3'K/AKT cascades. Moreover, these observations suggest that *HER-2/neu*, HRG, and CCN1 share similar signaling pathways, which may also account for the biological effect of CCN1 on the regulation of VEGF. Using U0126, a specific inhibitor of MEK-1 and MEK-2 activities (24), we investigated whether interfering with the MAPK signaling pathway would inhibit CCN1-enhanced $VEGF_{165}$ secretion. U0126 treatment only affected MAPK signaling, as indicated by the complete inhibition of P-MAPK without affecting P-AKT, and concurrently decreased CCN1-enhanced secretion of $VEGF_{165}$. In fact, the inactivation of the MAPK signal transduction pathway by U0126 resulted in a dramatic down-regulation of $VEGF_{165}$ secretion in CCN1-overexpressing MCF-7/C2-6 and MCF-7/C2-9 transfectants (Figure 6). To evaluate the involvement of PI-3'K/AKT signaling in CCN1-enhanced secretion of $VEGF_{165}$ secretion, we tested whether LY294002, a competitive inhibitor of PI-3'K activity, would block CCN1-enhanced secretion of $VEGF_{165}$. LY294002 exposure only affected PI-3'K signaling, as indicated by the complete inhibition of Ser-473 AKT without affecting P-MAPK, and concomitantly induced a significant decrease of $VEGF_{165}$ secretion (Figure 6). These results indicate that, down-stream of CCN1, MAPK-dependent signaling and PI-3'K-dependent AKT activity are participating in the transduction of signals that result in the increased secretion of $VEGF_{165}$ found in CCN1-overexpressing MCF-7 cells. Nonetheless, our results reveal that the MAPK signaling, as compared with PI-3'K/AKT pathway, plays a more important role in the up-regulation of $VEGF_{165}$ secretion of CCN1-overexpressing breast cancer cells.

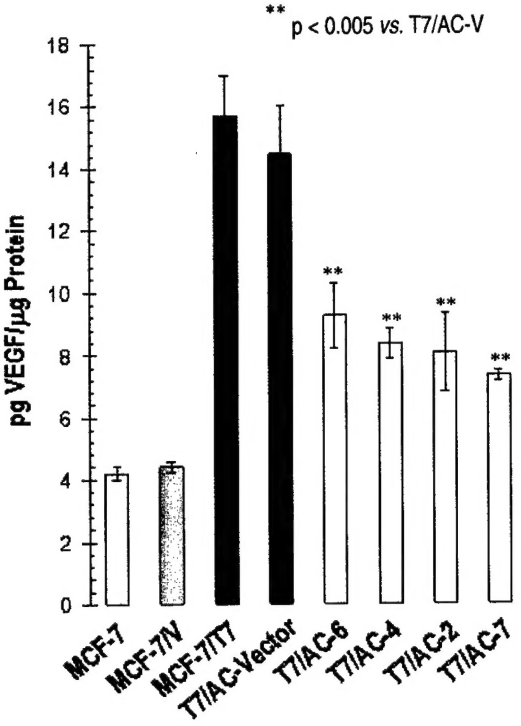


Figure 7. $VEGF_{165}$ secretion levels in antisense-CCN1 HRG-overexpressing MCF-7/T7 transfectants.

6. Antisense Down-Regulation of CCN1 (CYR61) Decreases VEGF secretion independently of HRG-induced activation of *HER-2/neu*. In the light of above observations, we hypothesized that CCN1 may be an angiogenic-promoting factor that acts independently of HRG overexpression. In this scenario, blockade of CCN1 expression will result in reduction of $VEGF_{165}$ secretion in cells that express high levels of HRG, such as the MCF-7/T7 transfectants. To block CCN1 expression, a eukaryotic expression vector was constructed with the CCN1 cDNA oriented from 3' to 5' end, that is, in an antisense direction, and subsequently transfected into HRG-overexpressing MCF-7/T7 clone. Several CCN1 antisense (T7/AC) clones were isolated and the presence of antisense CCN1 mRNA was confirmed by the RNAse protection assay (data not shown). Also generated were multiple clones of vector-transfected MCF-7/T7 (T7/AC-V) cells, all of which behaved similarly to the wild-type MCF-7/T7 clone. Four MCF-7/AC clones (T7/AC-2, T7/AC-4, T7/AC-6, and T7/AC-7) and one vector clone (T7/AC-V1) were characterized further. Conditioned media from AC-2, AC-4, AC-6, and AC-7 were collected and the secretion levels of $VEGF_{165}$ were determined as previously described. Figure 7 demonstrates the effect of antisense down-regulation of CCN1 expression on the basal level of $VEGF_{165}$ in the HRG-overexpressing MCF-7/T7 transfectants. Interestingly, blockade of CCN1 expression in HRG-overexpressing T7 transfectants led to dramatic decreases in HRG-induced $VEGF_{165}$ stimulation. However, the blockade of CCN1 expression in MCF-7 cells engineered to overexpress HRG was not able to reduce $VEGF_{165}$ secretion to the basal level observed in the wild type or MCF-7/V cells. Importantly, the levels of *HER-2/neu* receptor expression and its phosphorylation status were unchanged in AS-CCN1 transfectants when compared with those found in T7 and T7/AC-V controls (data not shown). We can then postulate that CCN1 is *necessary* for the optimal stimulation of $VEGF_{165}$ secretion in HRG-overexpressing breast cancer cells.

7. CCN1 receptor integrin $\alpha_v\beta_3$ plays a role in HRG- and CCN1-stimulated secretion of $VEGF_{165}$ trough MAPK signaling. CCN1 is a ligand of the integrin $\alpha_v\beta_3$ (14-17). We have previously reported that a functional blocking antibody agains $\alpha_v\beta_3$ is capable of blocking HRG induction of the aggressive phenotypes of the breast cancer cells (5). It has been shown that CYR61 is an angiogenic ligand for $\alpha_v\beta_3$ integrin receptor (14-17). Thus, we have proposed that CCN1 can mediate tumor growth and angiogenesis of breast cancer cells in either autocrine or paracrine manner through its binding to the $\alpha_v\beta_3$ integrin receptor (7, 12). Here, we decided to examine whether CCN1-induced secretion of $VEGF_{165}$ was associated with an increased integrin signaling. We compared six novel RGD peptidomimetic compounds having different integrin selectivity (Table 1), for their inhibition of $VEGF_{165}$ secretion in CCN1- and HRG-overexpressing human breast cancer cells. Our results showed that S-247, the RGD antagonist with the highest affinity for $\alpha_v\beta_3$ integrin, significantly decreased $VEGF_{165}$ secretion in CCN1-overexpressing MCF-7 cells (Figure 8). Similarly, a significant inhibitory effect on $VEGF_{165}$ secretion was induced by S-247 integrin antagonist in HRG-overexpressing MCF-7/T7 transfectants. No significant effects were observed when RGD-based "peptidomimetics" directed against other integrins, such as II_bIII_a, were used.

Among a variety of signaling cascades, the phosphorylation of focal adhesion kinase (FAK) and activation of the PI-3'K/AKT signaling pathway have been demonstrated to be involved in $\alpha_v\beta_3$-mediated adhesion, migration, and cell survival (25-27). Accordingly, we have recently demonstrated that CCN1 overexpression in MCF-7 cells induces a

prominent increase in the expression of FAK and significantly activates the PI-3'K/AKT signaling pathway in MCF-7 cells (12). Surprisingly, incubation of CCN1- and HRG-overexpressing MCF-7 cells with S-247 did not decrease the phosphorylation of either FAK or AKT (data not shown). Because integrins are involved in numerous pathways, S-247 is likely to inhibit signaling independently of FAK or AKT. Remarkably, we recently found that S-247 completely abolished MAPK hyperactivation in MCF-7 cells engineered to overexpress CCN1 or HRG (data not shown). Although CCN1 binds to several integrins, so far only the $\alpha_v\beta_3$ integrin has been shown to play a major role in breast cancer tumor vascularization and progression (13). Our current results further imply that CCN1 is a downstream effector of HRG-promoted breast cancer neovascularization through its ability to enhance $VEGF_{165}$ secretion likely *via* activation of a $\alpha_v\beta_3$-MAPK signaling cascade in breast cancer cells.

Table 1. RGD antagonists and their IC_{50} values against $\alpha_v\beta_3$ and II_bIII_a integrin functions

RGD antagonist	IC_{50} $\alpha_v\beta_3$	IC_{50} II_bIII_a
SC56631	10 nM	9 nM
SC68448	0.8 nM	407 nM
S-247	**0.6 nM**	1290 nM
S-196	1.19 nM	54 nM
S-205	> 100000	1.36 nM
S-106	38.8 nM	3250 nM

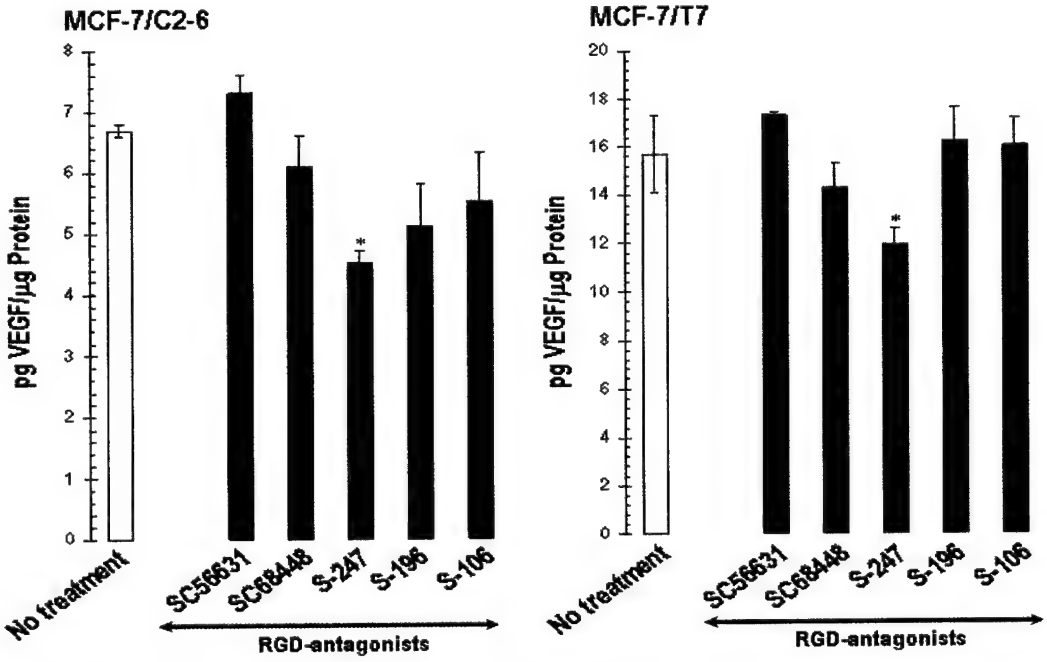


Figure 8. Effects of RGD antagonists on $VEGF_{165}$ secretion in CCN1- and HRG-overexpressing MCF-7 cells. CCN1-overexpressing MCF-7/C2-6 and HRG-overexpressing MCF-7/T7 transfectants were exposed to 1 µM SC56631, SC68448, S-247, S-196, and S-106 RGD-peptidomimetics for 48 h. $VEGF_{165}$ secretion was evaluated as described above.

KEY RESEARCH ACCOMPLISHMENTS

- High levels of HRG, independently of *HER-2/neu* overexpression, leads to up-regulation of $VEGF_{165}$ secretion in human breast cancer cells.
- HRG-stimulated secretion of $VEGF_{165}$ requires an autocrine action of HRG on *HER-2/neu*-dependent signaling. However, HRG-stimulated secretion of $VEGF_{165}$ does not require a nuclear localization of HRG.
- The growth factor-inducible immediate-early gene CCN1 (CYR61), a down-stream effector of HRG-induced breast cancer progression, is *sufficient* to induce up-regulation of $VEGF_{165}$ in human breast cancer cells in the absence of HRG and *HER-2/neu* overexpression.
- CCN1 (CYR61) enhances $VEGF_{165}$ secretion *via* activation of MAPK and PI-3'K/AKT signaling cascades.
- CCN1 (CYR61) is *necessary* for the maximal induction of HRG-dependent secretion of $VEGF_{165}$ in human breast cancer cells.
- The activation of a CCN1/$\alpha_v\beta_3$/MAPK signaling network could drive $VEGF_{165}$ secretion in CCN1- and HRG-overexpressing human breast cancer cells.

CONCLUSIONS

Among several angiogenic factors (VEGF, VEGF-related protein, VEGF-B, -C, -D, -E, placenta growth factor, and basic and acidic fibroblast growth factor), VEGF has been shown to be the major tumor angiogenic factor (28). VEGF can stimulate endothelial cell mitogenesis, and its overexpression has been detected in tumor cells, and was associated with high metastatic potential (28, 29). Additionally, inhibition of VEGF signaling has been shown to impair tumor growth and suppress tumor metastasis (30). The present study was designed to examine the HRG-dependent regulation of the VEGF secretory isoform, $VEGF_{165}$, in breast cancer cells that not overexpress the *HER-2/neu* oncogene. MCF-7 human breast cancer cells engineered to overexpress HRG offer the distinct advantage of studying ligand-*HER-2/neu* receptor activation in the absence of *HER-2/neu* overexpression. In this regard, our current analysis explored a uniquely different mechanism through ligand (HRG)-mediated transactivation of *HER-2/neu* signaling and up-regulation of $VEGF_{165}$ secretion. We used matched MCF-7 parent and HRG-transfected daughter human breast cancer cells, which differ in their HRG expression level, to evaluate the role of HRG on CDDP effectiveness. In addition, we performed parallel studies in retroviral HRG-infected MCF-7 cells to circumvent the possibility that phenomena due to effects other than HRG overexpression (*i.e.* clone specific) might be observed. Using this approach, we were able to directly compare $VEGF_{165}$ secretion in *HER-2/neu*-negative parental cells with low-expression of HRG to identical daughter cells with high-expression of HRG *in vitro*.

Several conclusions can be drawn from the results presented in this study: First, using a panel of breast cancer cell lines with constitutive *HER-2/neu* overexpression, HRG overexpression or engineered to stably overexpress HRG, we found that $VEGF_{165}$ secretion was significantly higher in HRG-overexpressing cells than in *HER-2/neu*-overexpressing and HRG-negative breast cancer cells. From the mechanistic point of view, it is clear that $VEGF_{165}$ secretion in HRG-overexpressing MCF-7 cells is not dependent upon *HER-2/neu* overexpression. In fact, the current data demonstrate a significant down-regulation of *HER-2/neu* protein levels following forced expression of HRG in MCF-7 cells. Thus, our results strongly suggest that HRG-induced transactivation of *HER-2/neu* signaling is *sufficient* to determine up-regulation of $VEGF_{165}$ secretion in breast cancer cells in the absence of *HER-2/neu* overexpression.

Second, we provide evidence that the pro-angiogenic functions of HRG can be attributed to different structural domains of the protein. We demonstrate that the deletion of both the N-terminus sequences (a putative nuclear localization signal –NLS-) and the cytoplasmic domain of HRG protein in the HRG-M4 structural mutant completely abolish the up-regulation of $VEGF_{165}$ secretion promoted by the full-length HRG. Of note, we recently reported that HRG-M4 blocks the aggressive phenotype that has been associated to with the full-length HRG (18). Since the HRG-M4 protein is sequestered into a cellular compartment and is not secreted into the culture media, thus preventing its autocrine action and $p185^{HER\text{-}2/neu}$ phosphorylation, the data derived from MCF-7/HRG and MCF-7/HRG-M4 cells strongly suggest that HRG-dependent transactivation of *HER-2/neu* signaling is sufficient to induce a prominent up-regulation of $VEGF_{165}$ in human breast cancer cells. On the other hand, the deletion of the N-terminus sequences in the HRG-M1 mutant is not sufficient to reverse HRG-induced over-secretion of $VEGF_{165}$, likely because this mutant, similarly to the full-length HRG, does not deprive breast cancer cells of pro-angiogenic transduction pathways provided by the activation of *HER-2/neu* signaling.

Third, the high levels of $VEGF_{165}$ secretion on MDA-MB-231 breast cancer cells, a natural model of HRG overexpression, were significantly diminished by transfection with antisense HRG cDNA. Interestingly, we recently demonstrated that blockade of HRG overexpression did suppress the aggressive phenotype of MDA-MB-231 breast cancer cells by inhibiting cell proliferation, preventing anchorage-independent cell growth, and suppressing the invasive potential of these cells *in vitro* (20). More importantly, we observed a marked reduction in tumor formation, tumor size, and a lack of metastasis *in vivo* (20). From our current observations, it is reasonable to suggest that blockade of HRG expression inhibits tumorigenicity and abolishes the metastatic process by perhaps inhibiting a large cascade of molecular events, including the secretion of the angiogenic factor $VEGF_{165}$.

Since CCN1 (CYR61) is also induced in HRG-overexpressing breast cancer cells (5), and HRG promotes tumorigenicity in part *via* up-regulation of CCN1 (5), we further investigated whether CCN1 overexpression by itself was sufficient to bypass the previously observed *HER-2/neu* requirement for HRG-enhanced secretion of $VEGF_{165}$. Forced expression of CCN1 in *HER-2/neu*- and HRG-negative MCF-7 human breast cancer cells resulted in a

significant increase in the secretion of $VEGF_{165}$, and this association occurred in the absence of *HER-2/neu* activation. Therefore, CCN1 is *sufficient* to up-regulate breast cancer cell secretion of $VEGF_{165}$ in the absence of HRG and *HER-2/neu* overexpression in breast cancer cells. Interestingly, CCN1 overexpression in HRG- and *HER-2/neu*-negative MCF-7 cells was accompanied by activation of MAPK and PI-3'K/AKT signaling pathways. Inactivation of the MAPK signaling using the specific inhibitor U0126 completely prevented CCN1-induced secretion of $VEGF_{165}$. Pharmacological inhibition of PI-3'K activity partially reversed CCN1-stimulated $VEGF_{165}$ secretion. These results indicate that, down-stream of CCN1, MAPK and PI-3'K-dependent AKT activity are participating in the transduction of signals that result in the increased secretion of $VEGF_{165}$ found in CCN1-overexpressing MCF-7 cells. Moreover, these observations suggest that *HER-2/neu*, HRG, and CCN1 share similar signaling pathways, which may also account for the biological effect of CCN1 on the promotion of breast cancer aggressiveness. Interestingly, S-247, a potent antagonist of CCN1 receptor $\alpha_v\beta_3$ integrin, significantly decreased CCN1-induced secretion of $VEGF_{165}$ in MCF-7/CCN1 and MCF-7/HRG transfectants. The incubation of MCF-7/CCN1 transfectants with S-247 did not decrease the phosphorylation of either AKT or FAK or AKT. However, the functional blockade of $\alpha_v\beta_3$ integrin receptor completely abolished MAPK hyperactivation in MCF-7 cells engineered to overexpress CCN1 or HRG. Because integrins are involved in numerous pathways, S-247 is likely to inhibit $\alpha_v\beta_3$-dependent cellular signaling *via* MAPK but independently of FAK or AKT. Although the exact mechanism(s) by which CCN1 enhances HRG-stimulated $VEGF_{165}$ secretion is still unknown, it is tempting to postulate that CCN1-induced activation of $\alpha_v\beta_3$-MAPK signaling could be a new *HER-2/neu*-independent pathway involved in this phenotype. Importantly, the antisense down-regulation of CCN1 expression significantly decreased $VEGF_{165}$ secretion in HRG-overexpressing MCF-7/T7 transfectants. These results strongly suggest that a CCN1-dependent signaling through $\alpha_v\beta_3$ integrin may be *necessary* for the maintenance of high levels of $VEGF_{165}$ secretion in HRG-overexpressing human breast cancer cells.

In summary, our current data provide new evidences about the role of HRG and its down-stream effector CCN1 (CYR61) as pro-angiogenic factors in human breast cancer cells. From a clinical perspective, current and future antagonists of specific integrin heterodimers, such those used in this study directed against $\alpha_v\beta_3$, or more specific anti-HRG and anti-CCN1 strategies, may have the potential to suppress tumorigenicity and metastasis of HRG- and CCN1-overexpressing breast carcinomas by decreasing VEGF-dependent breast cancer angiogenesis.

REPORTABLE OUTCOMES

ABSTRACTS/PRESENTATIONS

Javier Abel Menendez, Inderjit Mehmi, Ella Atlas, Miaw-Sheue Tsai, and **Ruth Lupu**. "The Angiogenic Factor CYR61, a Downstream Effector of Heregulin, Protects Breast Cancer Cells from Paclitaxel-induced Cell Death Through Integrin $\alpha_v\beta_3$" Abstract #366. **European Journal of Cancer** Vol. 38, Supplement 7, p. 108. November 2002.
14th EORTC-NCI-AACR Symposium on "Molecular Targets and Cancer Therapeutics"
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Ruth Lupu, and **Javier A. Menendez**. "Overexpression of the Angiogenic Factor CYR61 Protects Human Breast Cancer Cells from Taxol-induced Cell Death: Involvement of the $\alpha_v\beta_3$/Focal Adhesion Kinase/Phosphatidylinositol 3'-kinase/AKT Kinase Pathway". **Tumor Progression Control and Hormones (non-steroidal) Session** of the **International Congress on Hormonal Steroids and Hormones and Cancer.**
Fukuoka, Japan, October 21-25, 2002.

Javier A. Menendez, Inderjit Mehmi, Ella Atlas, Miaw-Sheue Tsai, David Griggs, and **Ruth Lupu**. "Overexpression of the Angiogenic Factor CYR61 Protects Human Breast Cancer Cells from Taxol-induced Cell Death: Involvement of the $\alpha_v\beta_3$/Focal Adhesion Kinase/Phosphatidylinositol 3'-kinase/AKT Kinase Pathway" **International Journal of Molecular Medicine.** November 2002.
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Javier A. Menendez, Inderjit Mehmi, David Griggs, and **Ruth Lupu**. "The angiogenic factor CCN1 (CYR61) protects breast cancer cells from Taxol-induced cell death"
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September 17-21. Chicago, Illinois, 2003.

MANUSCRIPTS

Javier A. Menendez, Inderjit Mehmi, David G. Griggs, and **Ruth Lupu**. "The Angiogenic Factor CYR61 in Breast Cancer: Molecular pathology and Therapeutic Perspectives" ***Endocrine-Related Cancer*** 10 (2): 141-152, 2003. Review.

Javier A. Menendez, Inderjit Mehmi, Luciano Vellon, Poh K. Teng, Vishal A. Verma, David Griggs, and **Ruth Lupu**. "The Angiogenic Factor CCN1 (CYR61) Induces Breast Cancer Cell Resistance to Paclitaxel (Taxol®) through Integrin $\alpha_v\beta_3$-dependent Activation of p42/44 MAPK Signaling Pathway" (2003) *Manuscript in preparation.*

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After this second year training period of the DOD BCRP post-doctoral traineeship I have developed and mastered several essential techniques in biochemistry, cell biology, and molecular biology, which is evident in the research accomplishments described above. In addition, it has offered me tremendous opportunities to establish interesting collaborations with other researchers in the field of human breast cancer. With the support from DOD, the proposed subject shall enhance our understanding at both the cellular and molecular levels of breast cancer progression, and could result in new molecular anticancer therapies based on blockade of HRG, CCN1, and/or the integrin receptor $\alpha_v\beta_3$.

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OFFPRINTS

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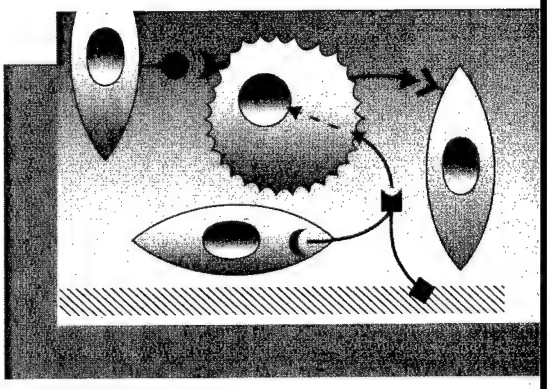

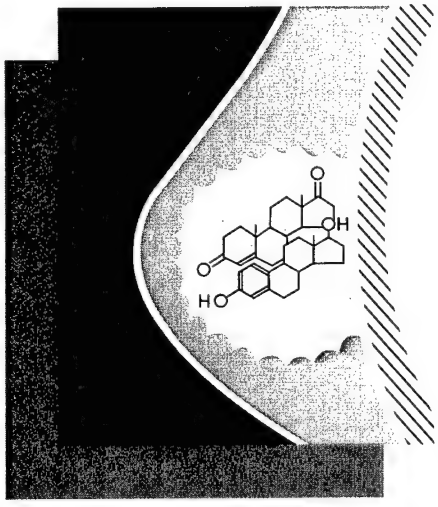

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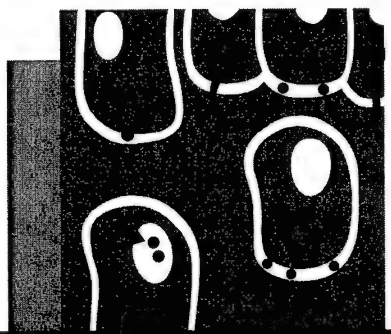

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The angiogenic factor CYR61 in breast cancer: molecular pathology and therapeutic perspectives

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Abstract

CYR61 (CNN1), a member of the cysteine rich 61/connective tissue growth factor/nephroblastoma overexpressed (CYR61/CTFG/NOV) family of growth regulators (CNN), is a pro-angiogenic factor that mediates diverse roles in development, cell proliferation, and tumorigenesis. We have recently shown that CYR61 is overexpressed in invasive and metastatic human breast cancer cells. Accordingly, elevated levels of CYR61 in breast cancer are associated with more advanced disease. Unfortunately, the exact mechanisms by which CYR61 promotes an aggressive breast cancer phenotype are still largely unknown. This review examines the functional role of CYR61 in breast cancer disease, presenting evidence that CYR61 signaling may play a major role in estrogen- as well as growth factor-dependent breast cancer progression. We also emphasize the functional significance of the molecular connection of CYR61 and its integrin receptor $\alpha_v\beta_3$ enhancing breast cancer aggressiveness. Moreover, we describe experimental evidence that establishes a novel role for CYR61 determining the protection of human breast cancer cells against chemotherapy-induced apoptosis through its interactions with the integrin receptor $\alpha_v\beta_3$. All these findings delineate a new noteworthy function of a CYR61/$\alpha_v\beta_3$ autocrine–paracrine signaling pathway within both angiogenesis and breast cancer progression, which would allow a dual anti-angiogenic and anti-tumor benefit with a single drug.

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Introduction

Steroid hormones closely regulate the growth of human breast cancer cells. Thus, anti-estrogen therapy is widely considered as the first-line therapeutic choice for the management of estrogen receptor (ER)-positive breast cancer. However, aggressiveness of human breast cancer is often related to the ability of the cells to overcome estrogen (E2) requirements for growth, and in most cases to acquire anti-estrogen resistance (Nicholson & Gee 2000). Thus, after the initial stages of breast cancer progression, tumors frequently acquire resistance to E2 with concurrent amplification and/or dysregulation of growth factors/growth factor receptor. The mechanisms that enable the progression from E2 dependence to E2 resistance remain poorly defined.

Within this context, we have shown previously that expression of heregulin (HRG), a growth factor that activates the erbB-2/3/4 receptor network signaling, is closely associated with an invasive breast cancer phenotype (Cardillo *et al.* 1995, Atlas *et al.* 2003). We have further demonstrated that HRG induces breast cancer progression, as determined by the loss of ER function and E2 response, tumorigenicity, invasion, and metastasis (Lupu *et al.* 1995, 1996, Tang *et al.* 1996, Atlas *et al.* 2003). As a part of our efforts to describe gene(s) directly involved in HRG-induced breast cancer aggressiveness, we recently isolated and identified CYR61, an angiogenic factor that is differentially expressed in ER-negative, HRG-positive breast cancer cells (Tsai *et al.* 2000). CYR61 (CCN1), an extracellular matrix-associated protein of the cysteine rich 61/connective tissue growth factor/nephroblastoma overexpressed (CCN) family which also includes CCN2 (CTFG), CCN3 (NOV), CCN4 (WISP-1), CCN5 (WISP-2), and CCN6 (WISP-3), is a product of an immediate early gene that mediates cell adhesion, induces

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cell migration, enhances growth factor-induced DNA synthesis in fibroblasts and endothelial cells, stimulates chemotaxis of fibroblasts and endothelial cells, and increases chondrogenesis in mesenchymal cells (O'Brien & Lau 1992, Frazier *et al.* 1996, Kireeva *et al.* 1996, 1997, 1998, Wong *et al.* 1997, Babic *et al.* 1998, Kolesnikova & Lau 1998, Chen *et al.* 2001). Significantly, expression of CYR61 enhances neovascularization and tumor formation of human tumor cells in immunodeficient mice (Babic *et al.* 1998, 1999, Xie *et al.* 2001*a*, Tsai *et al.* 2002*a*).

CYR61 is a novel ligand for integrins, and signaling through integrin receptors such as $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha IIb\beta_3$, and $\alpha_6\beta_1$ may explain many, if not all, of the known diverse functions of CYR61 (Kireeva *et al.* 1998, Jedsadayanmata *et al.* 1999, Chen *et al.* 2000, Grzeszkiewicz *et al.* 2001). Although CYR61 binds to several integrins, so far only the integrin $\alpha_v\beta_3$ has been shown to play a major role in breast cancer tumor neovascularization and progression (Meyer *et al.* 1998, R Lupu *et al.* unpublished data). More importantly, it has been demonstrated that overexpression of $\alpha_v\beta_3$ is a marker for poor prognosis in breast cancer (Gasparini *et al.* 1998). In this regard, we have previously reported that a functional blocking antibody against $\alpha_v\beta_3$ is capable of inhibiting HRG induction of the aggressive phenotypes of breast cancer cells (Tsai *et al.* 2000). Excitingly, we have recently found that forced expression of CYR61 in HRG-negative MCF-7 human breast cancer cells led to the up-regulation of the integrin receptor $\alpha_v\beta_3$ (Menéndez *et al.* 2002, 2003). Furthermore, our more recent experimental data have established a novel role for CYR61 overexpression in determining protection of human breast cancer cells against paclitaxel (Taxol) (Menéndez *et al.* 2002, 2003), a microtubule-targeting drug that is among the most effective agents in the treatment of advanced breast cancer, refractory or non-responsive to endocrine manipulation (Seidman *et al.* 1995, Perez 1998). In addition to its effect on angiogenesis, CYR61 may play, therefore, an important role transmitting survival signals in either an autocrine or paracrine manner through a CYR61/$\alpha_v\beta_3$-primed regulatory loop in the absence of HRG overexpression.

This article provides a body of evidence demonstrating that aberrant expression of CYR61 promotes breast tumorigenesis and cancer progression by participating in the escape from anti-hormone control of cell growth. Also, we emphasize the functional significance of CYR61 and its mechanisms of action through the integrin receptor $\alpha_v\beta_3$ in human breast cancer cells. Ultimately, we speculate that targeting the CYR61/$\alpha_v\beta_3$-enhanced cell survival mechanism may prove therapeutically efficacious in the prevention or treatment of breast cancer.

Molecular pathology of CYR61 in breast cancer

CYR61, a molecular definition

CYR61 is a cysteine-rich, heparin-binding protein that is secreted and associated with the cell surface and the extracellular matrix (Yang & Lau 1991), biochemical features that resemble the Wnt-1 proto-oncogene and a number of known growth factors (Yang & Lau 1991). The human CYR61 cDNA encodes a protein 379 amino acids in length with a molecular mass of 42 kDa, and the gene is located on the short arm of chromosome 1 (1p22–31) (Charles *et al.* 1991, Jay *et al.* 1997). Of note, abnormalities of chromosome 1p have correlated with ER negativity and a poor prognosis in breast cancer (Hainsworth *et al.* 1992), and other malignancies (Simon *et al.* 1991, Shin *et al.* 1993, Gehring *et al.* 1995). CYR61 was originally identified by differential hybridization screening of a cDNA library of serum-stimulated BALB/c 3T3 fibroblasts (O'Brien *et al.* 1990). CYR61 is not expressed in quiescent fibroblasts, but it is transcriptionally activated within minutes after stimulation by serum, epidermal growth factor, basic fibroblastic growth factor (bFGF), platelet-derived growth factor, transforming growth factor-β, and 12-O-tetradecanoylphorbol 13-acetate (TPA) (Lau & Nathans 1985, 1987, Nathans *et al.* 1988, O'Brien *et al.* 1990, Tsai *et al.* 2002*b*).

CYR61 belongs to the CCN gene family of angiogenic regulators, which consists of CCN1 (CYR61), CCN2 (CTFG), CCN3 (NOV), CCN4 (WISP-1), CCN5 (WISP-2), and CCN6 (WISP-3) (Lau & Lam 1999, Brigstock *et al.* 2003). All CCN proteins, including CYR61: (1) display a high degree of conservation among family members and across species; (2) are cysteine rich and structurally similar to extracellular matrix-associated molecules; (3) are composed of multifunctional modular domains with similar sequence homologies to insulin-like growth factor-binding protein, to von Willebrand factor type C repeat, to thrombospondin type 1 repeat, and to growth factor cysteine knots; and (4) have been shown to mediate functions as diverse as cell proliferation, migration, adhesion, cell survival, differentiation, and extracellular matrix formation. They also regulate more complex processes, such as angiogenesis and tumorigenesis (for reviews see Brigstock 1999, Lau & Lam 1999, Perbal 2001).

Clinical relevance of CYR61 in human breast cancer

Increasing efforts have been devoted to defining the clinical relevance of cellular proteins that play a role in breast cancer progression. This interest has been particularly focused on proteins that are potential clinical indicators of disease prog-

nosis. To determine whether expression of CYR61 may have any clinical relevance in breast cancer, we performed a pilot study using Western blot analysis on proteins extracted from paraffin sections of breast tumor biopsies. In about 40% of the tumor specimens, all of which were ER-negative invasive breast carcinomas, we found high expression of the CYR61 protein (Tsai *et al.* 2000). Additional studies revealed that CYR61 was present in about 30% of breast tumor specimens as determined by immunohistochemistry, while no staining was observed in normal components of the biopsies (Tsai *et al.* 2000). Using Northern blot analysis, Xie *et al.* (2001*b*) also found CYR61 to be highly expressed in 36% of primary breast tumors. Sampath *et al.* (2001) showed that CYR61 was overexpressed in 70% of breast cancer patients with infiltrating ductal carcinomas of the breast and CYR61 was localized exclusively to hyperplastic ductal epithelial cells. More recently, overexpression of CYR61 was found in 39% of primary breast cancers using quantitative real-time PCR assay (Xie *et al.* 2001*b*). In this study, a significant correlation was found between elevated levels of CYR61 and advance stage and size of the primary tumor and lymph node involvement at the time of removal of the primary tumor.

All these results, taken together, indicate that prominent expression of CYR61 may play a role in the process of breast cancer development and might serve as a valuable tool for monitoring the tumor status of breast cancer patients. Further studies, however, should attempt to determine whether measurement of CYR61 at diagnosis could provide prognostic data and suggest those tumors that might be responsive to therapy. Incidentally, our group has extensively analyzed the aberrant signaling pathways that are activated by CYR61 overexpression which, hopefully, may offer new valuable therapeutic targets in breast cancer.

Expression and regulation of CYR61 in human breast cancer cell lines

We have demonstrated that expression of the growth factor HRG, a growth factor that activates the erbB-2/3/4 receptor network signaling, is highly associated with an aggressive progression of breast cancers to hormone independence, antiestrogen resistance, invasion, and metastasis (Lupu *et al.* 1995, 1996, Tang *et al.* 1996, Hijazi *et al.* 2000). To identify genes that are involved in the HRG induction of breast cancer progression, a number of genes were isolated and cloned by differential expression in the MDA-MB-231 model, a human breast cancer cell line that naturally overexpresses HRG. Sequence and homology analyses evidenced that one of these genes was the human homologue of a mouse immediate early response gene, *Cyr61*. CYR61 was highly and selectively expressed (a 5- to 25-fold increase in the CYR61 mRNA level) in MCF-7 human breast cancer cells engineered to overexpress HRG (HRG-transfected MCF-7 clones T2, T6, T7, and T8) (Tang *et al.* 1996, Harris *et al.* 1998), but was almost undetectable in vector-transfected MCF-7 cells (Tsai *et al.* 2000). HRG-positive MDA-MB-231 cells also expressed high levels of CYR61 mRNA. Western blot and immunohistochemical analyses using an anti-CYR61 polyclonal antibody demonstrated that the CYR61 protein was selectively up-regulated in human breast cancer cells forced to overexpress HRG. Moreover, when the basal level of CYR61 expression was examined in a panel of human breast

Table 1 CYR61 overexpression is associated with ER-negativity and aggressiveness of human breast cancer cell lines.

Cell line	CYR61	HRG	ER	Invasive *in vitro*	Metastatic *in vivo*	$\alpha_v\beta_3$[b]
MCF-7	–[a]	–	++++	–[c]	–	+/–
T47D	–	–	++	–[c]	–	–
BT-474	–	–	++	–[c]	–	–
MDA-MB-175	–	+/–[d]	+	–[c]	–	ND
ZR-75B	–	+/–[d]	+	–[c]	–	–
MDA-MB-468	+	–	–	+[e]	–	–
SK-Br3	+	–	–	+[e]	–	–
MDA-MB-157	++	++	–	+	ND	ND
MDA-MB-436	+++	+++	–	+++	ND	ND
BT-549	+++	+++	–	++++	+	ND
MDA-MB-231	++++	++++	–	++++	+	+++
MDA-MB-435	++++	++++	–	++++	+	+++
Hs578T	++++	++++	–	++++	+	+++
MCF-7/HRG	+++	+++	–/+	++++	+	++
MCF-7/CYR61	++++	ND	–/+	++++	–	++

[a]–indicates no expression; the number of plus signs (+) indicates the increase in expression.
[b]The integrin $\alpha_v\beta_3$ expression is based on results from Kireeva *et al.* (1996), and our preliminary data. ND, not determined.
[c]Cells require E2 for invasion *in vitro* and growth *in vivo* and never metastasize *in vivo*.
[d]E2 induces expression of HRG.
[e]Cells require EGF or HRG to invade but never metastasize *in vivo*.

cancer cell lines, we found that CYR61 mRNA and protein were highly expressed in MDA-MB-231, Hs578T, BT549, and MCF-7/HRG cells, all of which are HRG-overexpressing and ER-negative cells (Tsai *et al.* 2000). Conversely, CYR61 levels were low or undetectable in cells that do not express HRG and are ER negative, including MCF-7, ZR75B, T47D, and BT-474 cells (Tsai *et al.* 2000). These data are summarized in Table 1. Our data undoubtedly demonstrated that high level of CYR61 expression tightly correlates with HRG overexpression and inversely correlates with ER expression. Moreover, the expression of CYR61 strongly correlates with vimentin expression, a known marker for invasiveness (Thompson *et al.* 1992), and is associated with the ability of breast cancer cells to invade *in vitro* and metastasize *in vivo*. Hence, there exists a tight correlation between HRG overexpression, CYR61 up-regulation, and breast cancer progression.

In our evaluation of CYR61 in breast tumor biopsies (Tsai *et al.* 2000), CYR61 expression was closely correlated with tumor progression and ER negativity. It has been recently shown that CYR61 overexpression is associated with more advanced breast cancer disease. However, although the sample number was relatively small, Xie *et al.* (2001*a*) showed a significant correlation between CYR61 expression and ER positivity, even though ER expression is known to be an indicator of good prognosis for breast cancer (Brown *et al.* 2000). Similarly, Sampath *et al.* (2001) suggested that increased levels of CYR61 in ER-positive breast tumors might contribute to E2-driven tumorigenesis *in vivo*. Some of these conflicting observations may be due to different methodologies used to quantify CYR61 expression, such as moderately sensitive Northern blots detecting CYR61 expression in malignant and non-malignant breast epithelium (Xie *et al.* 2001*a*) versus highly sensitive and breast epithelium-specific immunohistochemical analysis (Tsai *et al.* 2000). These somewhat mystifying results led us to use a systematic *in vitro* and *in vivo* approach assessing the actual relationship of CYR61 and ER-signaling pathways in human breast cancer cell growth and progression.

Previous studies showed that murine *Cyr61* is inducible by E2 and tamoxifen in the uterus of ovariectomized rats (Rivera-Gonzalez *et al.* 1998). However, it is not clear how CYR61 is regulated in human breast cancer, except that it has been indicated that CYR61 is E2 inducible, and that tamoxifen inhibited its mRNA expression in human breast cancer cells (Xie *et al.* 2001*a*). Therefore, we investigated in more detail how CYR61 expression is regulated in both ER-positive and ER-negative breast cancer cells (Tsai *et al.* 2002*b*). We were the first group to establish that expression of CYR61 at both the mRNA and protein levels was inducible by E2 in E2-dependent breast cancer cells (Tsai *et al.* 2002*b*). Moreover, we demonstrated that treatment of E2-depleted cells with the anti-estrogens tamoxifen and ICI 182 780 caused a marked up-regulation of CYR61 mRNA. Tamoxifen, a well-known anti-estrogen, functions as an agonist and antagonist through both transcriptional activation domains (AF1 and AF2) of ER. ICI 182 780, a pure anti-estrogen, acts solely as an antagonist through the AF1 domain (McGregor & Jordan 1998). Interestingly, the combination of E2 with either anti-estrogen abrogated the activation of CYR61 gene expression. We observed that CYR61 protein expression was also up-regulated (over 10- to 20-fold) by E2, tamoxifen and ICI 182 780 in MCF-7 cells. Other important regulators of CYR61 expression in breast cancer cells that we found were the phorbol ester TPA, vitamin D, and retinoic acid. TPA caused positive regulation of CYR61 expression in ER-positive MCF-7 cells. Vitamin D induced a transient stimulatory effect on CYR61 gene expression. Finally, the differentiating agent, retinoic acid, down-regulated CYR61 expression in MCF-7 breast cancer cells. In cells that acquire E2 independence but still express ER, such as MCF-7 cells transfected with HRG (MCF-7/HRG), E2 induced CYR61 expression to a much lower extent; conversely, these agents no longer mediated the expression of CYR61 in MDA-MB-231 cells, which express high levels of endogenous CYR61. This finding was consistent with the phenotype of the MDA-MB-231 cell line, which is ER negative, E2 independent, anti-estrogen resistant, highly invasive, and metastatic *in vivo*.

Together, our results proved that the fold induction of CYR61 by E2 and/or anti-estrogen agents coincides with the endogenous levels of CYR61 expression and is inversely correlated with the levels of ER expression in human breast cancer cells. Moreover, our results were in agreement with our knowledge that CYR61 promotes tumor growth, and that anti-estrogen agents have a positive impact on breast cancer cells expressing low levels of CYR61 (ER-positive breast cancer cells); conversely, these agents have no significant effect on cells that express high levels of CYR61 (ER-negative breast cancer cells).

CYR61 is sufficient to promote acquisition of E2 independence and anti-estrogen resistance in human breast cancer cells

CYR61 is differentially expressed in HRG-positive, invasive, and metastatic human breast cancer cells (Tsai *et al.* 2000). Moreover, enhanced expression of CYR61 correlates with lack of ER expression. To determine whether ectopic expression of CYR61 alone, in HRG-negative breast cancer cells, is necessary and/or sufficient to confer some biological activities induced by HRG, such as loss of E2 response and acquisition of anti-estrogen resistance, MCF-7 human breast cancer cells, which are ER positive, E2 reponsive *in vitro*, and are growth inhibited by many anti-estrogen drugs (Nicholson *et al.* 1995), were engineered to overexpress CYR61. In anchorage-dependent assays, our breast cancer models of CYR61 overexpression showed a growth advan-

tage in E2-depleted media, having a three- to fivefold increase in growth as compared with control cells (Tsai *et al.* 2002). These results illustrate that overexpression of CYR61 provides a growth advantage to bypass the 'normal' E2 requirement for the proliferation of MCF-7 human breast cancer cells (Pratt & Pollak 1993, Tsai *et al.* 2002). Interestingly, CYR61-overexpressing MCF-7 cells were still responsive to E2, resembling one of the clinical phenotypes found in women suffering from breast cancer. When MCF-7/CYR61 cells were treated with tamoxifen or ICI 182 780, both anti-estrogens reduced only the growth induced by E2 in MCF-7/CYR61 cells. However, they were unable to block the E2-independent growth of the MCF-7/CYR61 cells, indicating that CYR61 provides a true growth advantage that cannot be inhibited by anti-estrogen agents. In other words, both anti-estrogens inhibited the growth of the cells only to the basal levels promoted by overexpression of CYR61, whereas they were not able to reduce cell growth to the same level achieved in the wild-type MCF-7 cells in the absence of E2. These *in vitro* experiments revealed, therefore, that CYR61 overexpression can stimulate cell growth of E2-dependent cells in the absence of E2s resulting in cells becoming E2 independent. On the other hand, E2s further enhances cell proliferation of CYR61-overexpressing cells, indicating that these cells, although independent of E2, are still responsive to E2. Therefore, it is likely that overexpression of CYR61 alone most probably accounts for the growth advantage observed in MCF-7/CYR61 cells in E2-depleted culture conditions.

A possible mechanism to acquire E2-independent and anti-estrogen-resistant phenotypes acts via the loss of ER expression and/or ER function. Within this context, we have observed that the basal level of ERα expression is markedly reduced (30–50%) in MCF-7/CYR61 cells (Tsai *et al.* 2002*a*). These results indicate that CYR61 expression correlates with the loss of ER expression, consistent with our previous finding that CYR61 expression is closely associated with tumor progression and ER negativity in tumor biopsies (Tsai *et al.* 2000). We next examined whether CYR61 promotes loss of ER function by assessing the regulation of several well-documented E2-responsive genes. Since we previously demonstrated that the loss of progesterone receptor (PgR) regulation by E2 attests for the loss of ER function (Saceda *et al.* 1996, Tang *et al.* 1996), our studies in MCF-7/CYR61 cells were focused on E2 regulation of ERα and PgR expression. Although the level of ERα expression was lowered, E2 exposure further down-regulated the expression of ERα in CYR61-overexpressing MCF-7 cells, and induced a marked up-regulation in PgR mRNA expression. Similarly, we observed E2-induced up-regulation of cathepsin D and trefoil factor 1 (TFF1, formerly pS2), which have been shown to be up-regulated by E2 in MCF-7 cells (Cavailles *et al.* 1988, Weaver *et al.* 1988). These data support the notion that ERα is still a functional receptor in MCF-7/CYR61 cells, although these cells are E2 independent and the level of ERα expression is lower than that in the parental cells.

CYR61 enhances a metastatic phenotype by promoting cell proliferation in soft agar, cell migration and invasion, and Matrigel outgrowth of breast cancer cells

Acquisition of a transforming phenotype is often correlated with the ability of cells to grow in an anchorage-independent fashion. It is well established that MCF-7 cells are not anchorage independent in the absence of E2. Colonies observed, if any, represent the background level for the colony formation assay. We and others observed that MCF-7 engineered to overexpress CYR61, in the absence of E2, formed large colonies in soft agar assays (Xie *et al.* 2001*a*, Tsai *et al.* 2002*a*). As expected, E2 exposure induced anchorage-independent growth of control MCF-7 cells, which was completely blocked by anti-estrogen. Interestingly, E2 exposure also slightly enhanced the colony formation in the CYR61-overexpressing MCF-7 cells, an E2-driven effect that was not reversed in the presence of the pure anti-estrogen ICI 182 780 (Tsai *et al.* 2002*a*). Xie *et al.* (2001*a*) noted that tamoxifen blocked the E2-stimulated colony formation in MCF-7/CYR61 cells. In contrast, we demonstrated that both tamoxifen and ICI 182 780 enhanced colony formation of MCF-7/CYR61 cells (Tsai *et al.* 2002*a*, R Lupu and M-S Tsai unpublished data). Nonetheless, all these studies clearly indicate that forced expression of CYR61 promotes anchorage-independent clonogenic growth of MCF-7 cells.

Using vitronectin-coated Boyden chambers, an *in vitro* assay to quantitate the invasive potential of tumor cells (Albini *et al.* 1987), Xie *et al.* (2001*a*) established that CYR61 stably transfected cell lines (MCF-12A and MCF-7) show significantly increased migration compared with the empty vector-transfected cells. In our experiments, we addressed the question whether CYR61 is a direct downstream regulation of HRG-induced metastatic properties in human breast cancer cells. For these studies we used HRG-transfected MCF-7 cells (Tang *et al.* 1996, Atlas *et al.* 2003), which have been shown to migrate through collagen in a Boyden chamber assay, and a CYR61-neutralizing antibody (Tsai *et al.* 2000). The anti-CYR61-neutralizing antibody inhibited migration of MCF-7/HRG cells in a dose-dependent manner. Similar results were observed in other invasive, HRG-overexpressing breast cancer cells, such as MDA-MB-231, Hs578T, and BT549. These studies demonstrated that CYR61 overexpression influences cell migration of MCF-7 cells, and suggest an association between the activation of CYR61 expression in human breast cancer and the invasive potential triggered by HRG. Recently, we established that MCF-7/CYR61 cells show extensive outgrowth in Matrigel (Tsai *et al.* 2002*a*), an *in vitro* assay that is frequently

employed as a reliable system to assess *in vitro* invasiveness of breast cancer cells (Sommer *et al.* 1994, Hijazi *et al.* 2000). Significantly, CYR61 overexpression promoted outgrowth of MCF-7 cells in the Matrigel matrix in the absence of E2, the colonies appearing large and irregular in shape. In contrast, control cells were not able to migrate through and proliferate in the Matrigel matrix even in the presence of E2, suggesting that CYR61 can induce an invasive phenotype of breast cancer cells in an E2-independent manner (Tsai *et al.* 2002*a*).

CYR61 promotes tumorigenesis and neovascularization

On the basis of our *in vitro* studies indicating that overexpression of CYR61 promotes anchorage-independent clonogenic proliferation in soft agar as well as cell migration and invasion, all of which are characteristics of an aggressive breast cancer phenotype, we envisioned that CYR61 overexpression should promote tumor development and neovascularization. Recently, we reported that MCF-7 cells transfected with CYR61 developed large and more vascularized tumors in ovariectomized nude mice (Tsai *et al.* 2002*a*). These tumors grew independently of hormonal stimulation, supporting our *in vitro* data suggesting that the MCF-7/CYR61 cells had a growth advantage in the absence of E2. The tumors developed for CYR61-overexpressing cells were highly vascularized. In agreement, overexpression of CYR61 promoted the expression of another important regulator of neovascularization, vascular endothelial growth factor (VEGF). Xie *et al.* (2001*a*) also described that MCF-7 breast cancer cells forced to overexpress CYR61 developed larger and more vascularized tumors in nude mice. Another interesting finding of this study is that overexpression of stably transfected CYR61 in the normal breast epithelial cell line MCF-12A, which does not normally express CYR61, resulted in tumor formation in nude mice. Taken together, these *in vitro* and *in vivo* findings suggest that prominent expression of CYR61 may facilitate transformation of breast tissue, and indisputably demonstrate that overexpression of CYR61 in MCF-7 cells promotes tumorigenesis in the absence of hormonal stimulation.

Therapeutic perspectives of CYR61 in breast cancer

CYR61 overexpression promotes breast cancer cell resistance to Taxol-induced cell death: involvement of the phosphatidylinositol 3′-kinase (PI3′-kinase)/ protein kinase B (AKT) pro-survival pathway

We have previously demonstrated that HRG-overexpressing cells showed a marked increase in sensitivity to the topoisomerase II inhibitors doxorubicin and etoposide (Harris *et al.* 1998). Interestingly, virtually every conventional cytotoxic anti-cancer drug has been 'accidentally' discovered to have anti-angiogenic effects in various *in vivo* models (Kerbel *et al.* 2000, Miller *et al.* 2001). Recently, the tumor cell microenvironment has been found to have a significant bearing on the survival of tumor cells following exposure to a wide variety of anti-neoplastic agents, prior to the acquisition of known drug resistance mechanisms. Accordingly, it has been recently demonstrated that some angiogenic factors such as VEGF and bFGF significantly reduced the potency of chemotherapy (Lissoni *et al.* 2000, Zhang *et al.* 2001, Tran *et al.* 2002). Because of the pro-angiogenic abilities of HRG and CYR61, we hypothesized that HRG could also act directly – or indirectly through CYR61 – as a survival factor for breast carcinoma cells modifying chemotherapy effectiveness. In this regard, we recently examined whether CYR61, in the absence of HRG overexpression, may play a role in the breast cancer cell responses to chemotherapy-induced damage. Significantly, we observed that CYR61 overexpression rendered MCF-7 cells resistant to paclitaxel (Taxol) in both anchorage-dependent and soft agarose colony-formation assays (Menéndez *et al.* 2002, 2003). Because apoptosis is the predominant mechanism of cytotoxicity induced by chemotherapeutic agents, we analyzed whether the failure of CYR61-overexpressing breast cancer cells to activate apoptosis may account for CYR61-promoted Taxol resistance. When MCF-7/CYR61 cells were examined for apoptosis-related parameters after Taxol exposure, no signs of the classical DNA laddering formation were observed in CYR61 transfectants compared with control cells. Accordingly, CYR61 overexpression induced a dramatic decrease in the number of TUNEL-positive cells compared with Taxol-treated control cells (Menéndez *et al.* 2002, 2003). It has been shown that Taxol-induced apoptosis involves a dose- and time-dependent accumulation of the tumor suppressor gene p53 and the inhibitor of cyclin-dependent kinases, $p21^{WAF1/CIP1}$ (Blasgosklonny *et al.* 1995, Giannakakou *et al.* 2001). Our preliminary experiments, however, noted a reduced ability of the MCF-7/CYR61 cells to induce p53 expression in response to Taxol exposure, suggesting that CYR61 overexpression could suppress Taxol-induced apoptosis by interfering with the function of wild-type p53 in human breast cancer cells (Menéndez *et al.* 2002, 2003).

Simultaneously to Taxol resistance, MCF-7/CYR61 cells showed cross-resistance to wortmannin and LY294002, two pharmacological inhibitors of the PI3′-kinase activity. Interestingly, we have demonstrated that CYR61-overexpressing MCF-7 cells undergo up-regulation of the PI3′-kinase/AKT kinase pro-survival pathway (Tsai *et al.* 2002*c*, Menéndez *et al.* 2002, 2003). Because several studies have recently indicated that alterations in the PI3′-

kinase/AKT signal transduction pathway can modulate cell sensitivity to Taxol (Mitsuuchi *et al.* 2000, Hu *et al.* 2002, MacKeigan *et al.* 2002), it is likely that a new important function of CYR61 in human breast cancer is the promotion of cell survival by activating anti-apoptotic signaling pathways such as those composed of PI3′-kinase and the serine/threonine kinase AKT. Accordingly, the protective effect of CYR61 against Taxol-induced cytotoxicity was abolished under culture conditions inhibiting PI3′-kinase activity (Menéndez *et al.* 2002, 2003). These findings establish a novel role for CYR61 in determining protection of human breast cancer cells against Taxol-induced apoptosis through the activation of the PI3′-kinase/AKT pro-survival pathway.

The 'CYR61–$\alpha_v\beta_3$ integrin connection': a new molecular therapeutic target in human breast cancer

Depending on the biological context and model system, CYR61 can induce disparate functions. However, most of the CYR61-promoted effects are mediated via its direct binding with the integrin receptor $\alpha_v\beta_3$. Thus, we speculated whether CYR61-enhanced breast cancer progression requires expression of the integrin receptor $\alpha_v\beta_3$ for its actions. Indeed, we observed that the level of $\alpha_v\beta_3$ was significantly augmented in MCF-7 engineered to overexpress HRG compared with control cells (Tsai *et al.* 2000). Moreover, we determined that blockade of this integrin receptor using LM609, a monoclonal antibody directed against $\alpha_v\beta_3$, dramatically blocked the Matrigel outgrowth of HRG-overexpressing breast cancer cells in a dose-dependent fashion (Tsai *et al.* 2000). These results indicated, for the first time, that the functional $\alpha_v\beta_3$ integrin is required for maintaining the invasive capacity of HRG-expressing cells, and that the aggressive phenotypes induced by HRG are mediated, in part if not entirely, through the interaction of CYR61 with integrin $\alpha_v\beta_3$.

Recently, we assessed whether CYR61, independent from HRG expression, affected the levels of the integrin receptor $\alpha_v\beta_3$. Use of a monoclonal anti-$\alpha_v\beta_3$ antibody demonstrated that, similarly to MCF-7/HRG cells, HRG-negative CYR61-overexpressing MCF-7 cells stained positively for $\alpha_v\beta_3$, whereas no significant staining was observed in control cells (Menéndez *et al.* 2002, 2003). To the best of our knowledge, this is the first indication showing that activation of $\alpha_v\beta_3$ expression in human breast cancer epithelial cells can be achieved solely by CYR61 overexpression, irrespective of HRG status. More importantly, the most recent data from our laboratory indicate that CYR61-promoted breast cancer cell survival and Taxol resistance are phenotypes associated with an increased $a_v\beta_3$ integrin signaling. Using SC56631, a previously characterized synthetic chemical peptide mimetic based upon the $\alpha_v\beta_3$ ligand, Arg-Gly-Asp (RGD motif) (Engleman *et al.* 1997), we observed a marked decrease in the cell viability of HRG- and CYR61-overexpressing breast cancer cells but not in HRG- and CYR61-negative control cells. Furthermore, sub-optimal doses of SC56631 completely abolished Taxol resistance in MCF-7/CYR61 cells, as Taxol-induced cytotoxicity returned to the basal level observed in CYR61-negative control cells. Moreover, the nature of the interaction between SC56631 and Taxol was shown to be synergistic in CYR61-overexpressing breast cancer cells (Menéndez *et al.* 2002, 2003). These results, together, strongly suggest that therapies depriving CYR61-overexpressing cells of their $\alpha_v\beta_3$ signaling dramatically decrease cell survival and chemoresistance.

Integrin $\alpha_v\beta_3$ has been implicated in the pathophysiology of malignant tumors. In addition to its expression on the surface of angiogenic endothelial cells, integrin $\alpha_v\beta_3$ is expressed on the surface of tumor cells in a variety of cancers. In breast cancer, the integrin $\alpha_v\beta_3$ characterizes the metastatic phenotype, as its expression is up-regulated in invasive tumors and distant metastases (Gasparini *et al.* 1998). Thus, independent of its role in tumor angiogenesis, the integrin $\alpha_v\beta_3$ is functionally implicated in the pathogenesis of breast cancer. Integrin signals are involved in diverse biological responses, including angiogenesis and tumor progression, as well as in a variety of cellular activities, including cell migration, proliferation, and survival. Of interest, integrin signaling has recently been shown to modulate cancer cell responses to chemotherapeutic agents (Aoudjit & Vuori 2001). Specifically, interactions between cell surface integrins and extracellular matrix components have been shown to be responsible for this phenomenon of innate drug resistance, which we have termed cell adhesion-mediated drug resistance (Damiano *et al.* 1999, Gilmore *et al.* 2000). Thus, signal transduction pathways initiated by integrin ligation may be potential bridge points for inhibiting cell survival during cytotoxic drug exposure. Among the signaling molecules involved in integrin-mediated cell survival is focal adhesion kinase (FAK), which becomes activated following integrin ligation and may in turn activate down-stream survival pathways such as those composed of PI3′-kinase and the serine/threonine kinase AKT (King *et al.* 1997, Gilmore *et al.* 2000, Lee & Juliano 2000). Likewise, we have recently demonstrated that CYR61 overexpression in MCF-7 human breast cancer cells induces a significant increase in the expression of FAK (Menéndez *et al.* 2002, 2003). On the basis of these studies it is tempting to postulate that up-regulation of CYR61 in breast cancer cells may drive breast cancer cell survival and chemoresistance by emitting a proliferative and/or survival input via the integrin receptor $\alpha_v\beta_3$ and FAK, which might integrate signals from CYR61 to the PI3′-kinase/AKT pro-survival pathway. Importantly, CYR61 can also promote cell survival of angiogenic endothelial cells through interaction with integrin $a_v\beta_3$ (Leu *et al.* 2002).

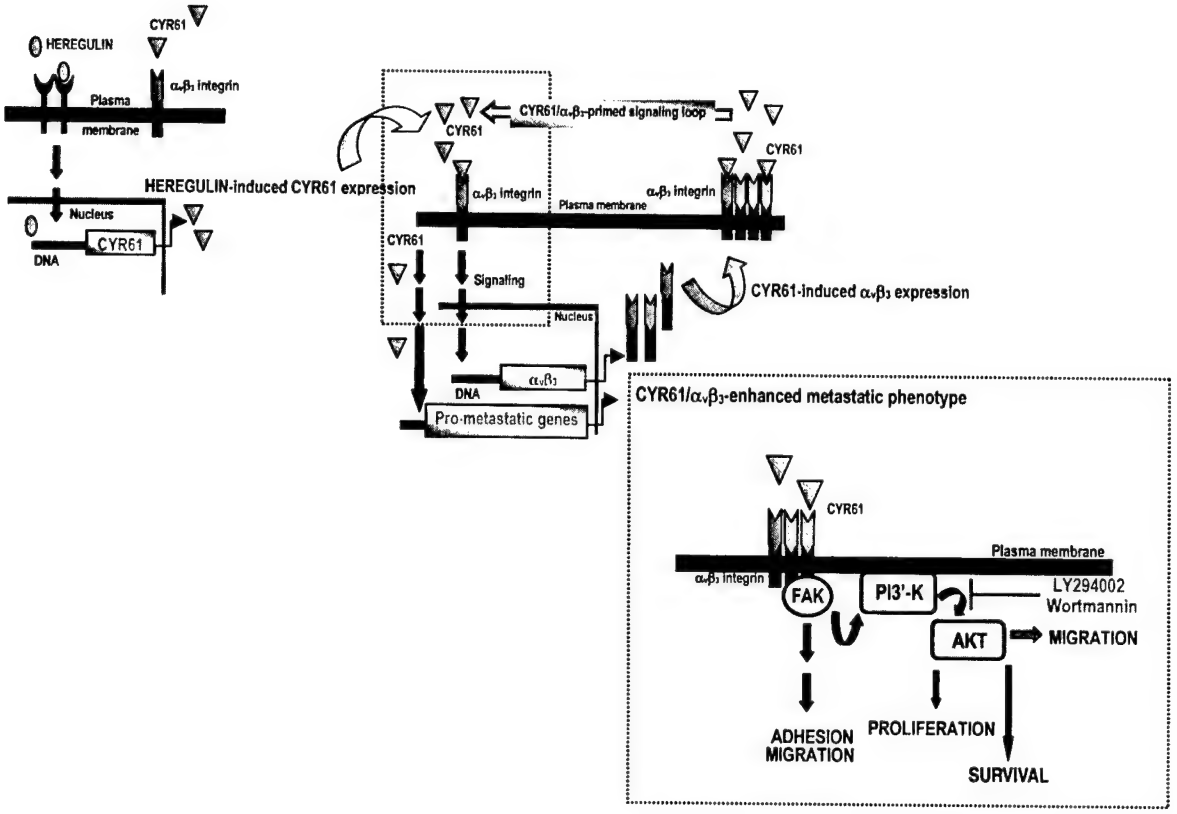

Figure 1 HRG-induced up-regulation of CYR61 may predispose breast tumor epithelial cells toward continued dysregulated proliferation and chemoresistance. The functional blocking of $a_v\beta_3$ integrin in HRG- and CYR61-overexpressing cells induces cytotoxicity, suggesting that CYR61-activated $\alpha_v\beta_3$ integrin signaling is involved in breast cancer cell survival. Since CYR61 overexpression by itself activates the expression of the CYR61 receptor integrin $\alpha_v\beta_3$, up-regulation of CYR61 in human breast cancer may co-ordinate a metastatic phenotype in an autocrine/paracrine manner by activating an $\alpha_v\beta_3$/FAK/PI3′-kinase/AKT kinase signaling.

The 'CYR61/$\alpha_v\beta_3$ connection' thus appears to provide a promising molecular target for breast cancer disease that should permit a potentially synergistic strike against the tumor and its supporting vasculature (Fig. 1).

Conclusions

Breast cancer often progresses from an E2-dependent, non-metastatic, anti-estrogen-sensitive phenotype to an E2-independent, anti-estrogen-resistant, highly invasive and metastatic phenotype. Our results have demonstrated that CYR61 is a tumor-promoting factor that also acts as a key regulator of breast cancer progression. Significantly, in our current studies we have demonstrated that the angiogenic factor CYR61 is sufficient (1) to induce E2 independence and anti-estrogen resistance, (2) to promote invasiveness *in vitro*, and (3) to induce tumorigenesis and neovascularization *in vivo*. Our results further suggest that signaling through $\alpha_v\beta_3$ integrin allows for the maintenance of the cell viability of human breast cancer cells treated with Taxol. Although the exact mechanism through which the angiogenic factor CYR61 promotes cell survival and Taxol resistance in breast cancer cells is still unknown, it is tempting to postulate that CYR61-induced activation of the $\alpha_v\beta_3$/FAK/PI3′-kinase/AKT pro-survival signaling could be a/the pathway involved in this phenotype. New anti-CYR61 and/or anti-$\alpha_v\beta_3$ therapeutic strategies may prevent vessel growth simultaneously rendering breast cancer cells more sensitive to Taxol-based chemotherapy.

In summary, our current approach indicates that activation of the CYR61/$\alpha_v\beta_3$ signaling network could drive breast cancer cells to escape hormonal requirements providing compensatory survival pathways that ultimately allow the acquisition of breast cancer chemoresistance. As knowledge of CYR61 involvement in breast cancer processes increases, the 'CYR61/$\alpha_v\beta_3$' connection in both angiogenic endothelial cells and tumor cells appears to be an increasingly attractive target for drug development (Fig. 2). Our data provide a starting point to exploit the potential anti-angiogenic and anti-tumor effects of highly specific $\alpha_v\beta_3$ inhibitors.

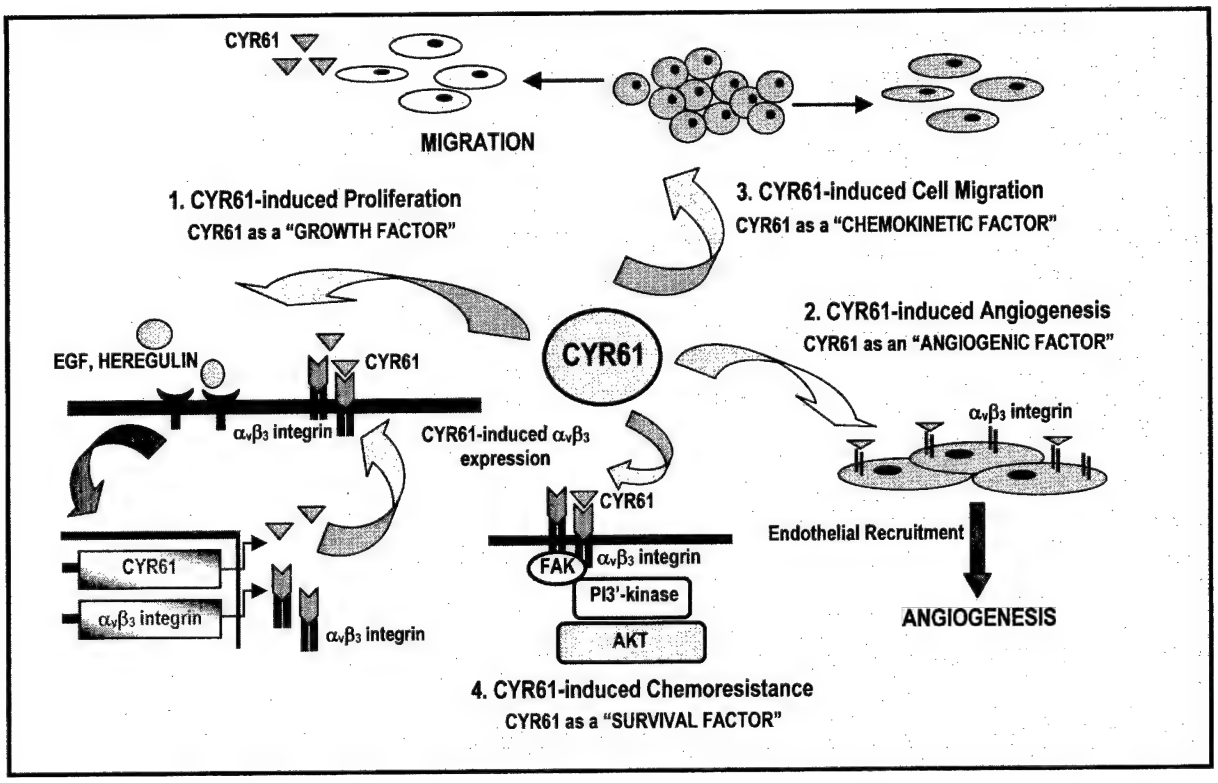

Figure 2 A new hypothetical model for the role of CYR61 in breast tumorigenesis and progression. Given that the angiogenic factor CYR61 is also a growth regulator, it is hypothesized that HRG-induced up-regulation of CYR61 in tumor epithelial cells may drive breast tumorigenesis and progression in several concerted modes: (1) by promoting tumor cell proliferation in an autocrine/paracrine fashion either augmenting growth factor bioactivity or emitting proliferative signals via $\alpha_v\beta_3$ integrin receptor, (2) by regulating endothelial recruitment tumor neovascularization in a paracrine fashion through an $\alpha_v\beta_3$-dependent mechanism, (3) by co-ordinating tumor epithelial cell migration as a chemokinetic factor, and (4) by increasing chemoresistance activating $\alpha_v\beta_3$/FAK/PI3′-kinase/AKT kinase pro-survival signaling.

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Coordinator: Dr. Ramón Colomer Bosch

2001

"TAXOL® EFFICACY AND FATTY ACIDS IN BREAST CANCER" (Bristol-Myers Squibb, Spain)
Position: *Associated Researcher*
Center: Medical Oncology Division, Hospital Universitario 12 de Octubre (Madrid, Spain)
Coordinator: Dr. Ramón Colomer Bosch

2001

"CLINICAL IMPLICATIONS OF OMEGA-3 FATTY ACIDS IN BREAST CANCER PROGRESSION"
(Comunidad Autonoma de Madrid, Spain)
Position: *Associated Researcher*
Center: Medical Oncology Division, Hospital Universitario 12 de Octubre (Madrid, Spain)
Coordinator: Dr. Ramón Colomer Bosch

2000-2001

"OMEGA-3 FATTY ACIDS IN THE PROGRESSION OF BREAST CANCER" (Sociedad Espanola de Oncologia Medica –SEOM-)
Position: *Associated Researcher*
Center: Medical Oncology Division, Hospital Universitario 12 de Octubre (Madrid, Spain)
Coordinator: Dr. Ramón Colomer Bosch

1999-2000

"FATTY ACIDS MODULATION OF ANGIOGENIC FACTORS AND ONCOGENES EXPRESSION" (FIS 96/0226)
Position: *Associated Researcher*
Center: Medical Oncology Division, Hospital Universitario 12 de Octubre (Madrid, Spain)
Coordinator: Dr. Ramón Colomer Bosch

1997-1998

"EXPERIMENTAL EVALUATION OF OLIVE OIL IN THE PREVENTION OF BREAST CANCER METASTASIS" (OLI96/2114)
Position: *Pre-Doctoral Fellow*
Center: Medical Oncology Division, Hospital Universitario 12 de Octubre (Madrid, Spain)
Coordinator: Dr. Ramón Colomer Bosch

1997-2000

RELEVANT RESEARCH EXPERIENCE (TECHNIQUES)

- **Basic Microbiological Techniques**: Bacterial culture and isolation. Bacteriophages culture, propagation, quantification and isolation. Isolation of viral genetic material.
- **Basic Recombinant DNA Techniques**: Culture and isolation of bacterial host. Clonation vectors (plasmids, M13 sequencing vectors and integration vectors). DNA isolation, sequencing and amplification.
- **Human Cell Culture.**
- **Electrophoresis and Western Blotting Techniques.**
- **Zimography Technique (Metalloproteinases detection).**
- **Proliferation, Adhesion and Migration/Invasion (Boyden chambers) assays.**
- **ELISA Technique.**
- **DNA and RNA isolation Techniques.**
- **PCR and RT-PCR Techniques.**
- **Chemosensitivity Assays (Anchorage-Dependent Cytotoxicity Assays, Anchorage-Independent Soft-Agar Colony Formation Assays).**
- **Pre-Clinical Analysis of Drug Interactions: Isobologram Analysis (Berenbaum Method) and Median-Effect Plot Analysis (Chou & Talalay Method).**
- **Southern Blotting Technique.**
- **Immunofluorescence Microscopy and Confocal Immunofluorescence Microscopy.**
- **Gene Retroviral Infections.**

- **Terminal Restriction Fragment (TRF) Length: Telomere Length.**
- **Telomerase Activity (TRAP assays): Telomeric Repeat Amplification Protocol.**
- **ERE assays.**

SCIENTIFIC MEETINGS and CONFERENCES

- ✓ "HARD DISEASES: CANCER AND AIDS" April-June, 1993
  Oviedo University, Spain.
- ✓ "A VACCINE AGAINST MALARIA: THE FUTURE OF SINTETIC VACCINES" 1995, Oviedo University, Spain.
- ✓ "THE BIOTECHNOLOGY AND ITS INDUSTRIAL APLICATIONS" 1995 and 1996
  Oviedo University, Spain.
- ✓ "VIII SYMPOSIUM on HUMAN FERTILITY" May 16, 1997
  Oviedo, Spain.
- ✓ "DEVELOPMENT OF NEW THERAPIES AGAINST CANCER" May 26, 1998
  José Casares Gil Foundation, Madrid, Spain.
- ✓ "10th AVANCED COURSE IN MEDICAL ONCOLOGY" June 1-4, 1998
  European School of Oncology; Madrid, Spain.
- ✓ "CELL CYCLE GENETIC ALTERATIONS IN NEOPLASTIC PROCESSES: BIOLOGICAL AND CLINICAL IMPLICATIONS" June 3, 1998
  José Casares Gil Foundation; Madrid, Spain.
- ✓ "1st INTERNATIONAL SYMPOSIUM ON LUNG CANCER: PROGRESS IN THE TREATMENT OF LUNG CANCER" June 5, 1998
  European Organization for Research and Treatment of Cancer (EORTC); Madrid, Spain.
- ✓ "VI ONCOGENES SYMPOSIUM" October 5-6, 1998
  Spanish Association for Cancer Research (ASEICA); Madrid, Spain.
- ✓ "PROTEIN AND NUCLEIC ACID CHEMISTRY" October 9, 1998
  Madrid, Spain.
- ✓ "NEW TARGETS IN THE TREATMENT OF CANCER: RATIONAL DEVELOPMENT OF ANTINEOPLASTIC DRUGS" October 13, 1998
  Madrid, Spain.
- ✓ "CANCER: BIOLOGY AND ANTI-CANCER AGENTS" October 29-30, 1998
  Spanish Association for Cancer Research (ASEICA)
  Granada, Spain.
- ✓ "MEDICAL ONCOLOGY TO PRIMARY ATTENTION" November 24-26, 1998
  Madrid, Spain.
- ✓ "II INTERNATIONAL SYMPOSIUM ON MOLECULAR DIAGNOSIS IN MEDICINE" November 26-27, 1998
  Madrid, Spain.

- ✓ "VIII CONGRESS SPANISH ASSOCIATION FOR CANCER RESEARCH (ASEICA)" and "I JOINT MEETING ASEICA-SPANISH ASSOCIATION FOR MEDICAL ONCOLOGY (SEOM)" April 19-21, 1999
  Sitges (Barcelona), Spain.
- ✓ "III INTERNATIONAL SYMPOSIUM CHANGES IN THE TREATMENT OF BREAST CANCER" June 2-4, 1999
  European Society of Medical Oncology (ESMO); Madrid, Spain.
- ✓ "VI ONCOGENES AND CANCER SYMPOSIUM" May 10-11, 2000.
  Madrid, Spain.
- ✓ "ANTI-PROLIFERATIVE EFFECT OF CANNABINOIDES" July 4, 2000.
  Madrid, Spain.
- ✓ "INHIBITORS of TRANSDUCTION SIGNALING PATHWAYS" February 8, 2001.
  Madrid, Spain.
- ✓ "92nd AMERICAN ASSOCIATION FOR CANCER RESEARCH (AACR) ANNUAL MEETING" March 24-28, 2001
  New Orleans, USA.
- ✓ "ENCUENTROS EN ONCOLOGIA" April 5-6, 2001
  Segovia, Spain.
- ✓ "IV Madrid BREAST CANCER CONFERENCE: CHANGES IN THE TREATMENT OF BREAST CANCER" June 7-9, 2001
  Madrid, Spain.
- ✓ "Simply SERMS: A view of the chemoprevention of breast cancer"
  50th Anniversary Cancer Research Laboratory Distinguished Lecture
  University of Berkeley, California, USA. November 27, 2001.
- ✓ "Cellular and Molecular Biology & Subcellular Structure: Postdoctoral Research Day"
  Lawrence Berkeley National Laboratory.
  University of Berkeley, California, USA. December 5, 2001.
- ✓ "93th AMERICAN ASSOCIATION FOR CANCER RESEARCH (AACR) ANNUAL MEETING"
  April 6-10, 2002
  San Francisco, USA.
- ✓ "ADVANCES IN TUMORAL BIOLOGY" November 6-8, 2002
  Oviedo, Spain.
- ✓ 44th ANNUAL CLINICAL CONFERENCE "MOLECULAR THERAPEUTICS FOR CANCER METASTASIS"
  The University of Texas MD Anderson Cancer Center
  Houston, Texas, USA. March 18-21, 2003.

ADDITIONAL ACADEMIC INFORMATION

DOCTORAL DEGREE PROGRAM in "BIOCHEMISTRY AND MOLECULAR BIOLOGY"
Complutense University, Madrid, Spain:

- "CELL CULTURES: TECHNIQUES AND APPLICATIONS"
  November 27, 1998-January 22, 1999
- "STRUCTURE OF PROTEINS"
  November 30, 1998-January 27, 1999
- "CELL PROLIFERATION, DIFFERENTIATION AND DEVELOPMENT"
  February 15-March 15, 1999
- "CHARACTERIZATION OF MACROMOLECULES"
  April 15-May 30, 1999
- "EXTRACELLULAR MATRIX: STRUCTURE AND FUNCTION"
  May 21-June 7, 1999
- "DINAMIC OF BIOLOGICAL MEMBRANES"
  November 24, 1999-February 24-2000
- "ONCOGENES AND CELLULAR MALIGNIZATION: TUMORAL MARKERS"
  May 10-11, 2000
- "LIPIDIC MESSAGERS IN SIGNAL TRANSDUCTION PATHWAYS"
  June 19-July 7, 2000

LANGUAGES

- SPANISH: Mother tongue.
- ENGLISH: Advanced.
- FRENCH: Advanced.

BIBLIOGRAPHY

A. PUBLICATIONS (Peer-reviewed publications in chronological order)

1. **J. A. Menéndez**, M. M. Barbacid, S. Montero, E. Sevilla, E. Escrich, H. Cortés-Funes and R. Colomer. "Effect of Gamma-Linolenic Acid and Oleic Acid on Paclitaxel Cytotoxicity in Human Breast Cancer Cells" ***European Journal of Cancer*** 37(3): 402-411, 2001. Article.

2. **Javier Abel Menéndez**, Eduard Escrich, Montserrat Solanas and Ramon Colomer. "Olive oil: A new strategy in the treatment of breast cancer" ***Mercacei*** 133-136. February 2001. (Spanish) Article.

3. **Javier Abel Menendez** and Ramon Colomer. "Omega-3 Fatty Acids and Breast Cancer Progression" Boletin de la Sociedad Española de Oncologia Medica 20: 27-29, 2001. (Spanish) Article.

4. Colomer, R., Montero, S., Ropero, S., **Menendez, J.**, Solanas, M., and Escrich, E. "The HER2 Oncogen as Example of Diagnostic and Therapeutic Progress in Breast Cancer" ***Revista de Senologia y Patologia Mamaria*** Vol. 14 (1): 8-19, 2001. (Spanish) Article.

5. Solanas, M., Hurtado, A., Costa, I., Moral, R., **Menendez, J. A.**, Colomer, R., and Escrich, E. "Effects of a High Olive Oil Diet on the Clinical Behavior and Histopathological Features of Rat DMBA-induced Mammary Tumors Compared With a High Corn Oil Diet" ***International Journal of Oncology*** 21(4): 745-753, 2002. Article

6. **J. A. Menéndez**, S. Ropero, M. M. Barbacid, S. Montero, E. Sevilla, M. Solanas, E. Escrich, H. Cortés-Funes and R. Colomer. "Synergistic Interaction Between Vinorelbine and Gamma-Linolenic Acid in Breast Cancer Cells" ***Breast Cancer Research and Treatment***. 72 (3): 203-219, 2002. Article.

7. **Javier Abel Menéndez**, Maria del Mar Barbacid, Sagrario Montero, Santiago Ropero, Eduard Escrich, Hernán Cortés-Funes and Ramon Colomer. "Effects of Dietary Fatty Acids on the Proliferation, Adhesion and Metastatic Potential of Breast Cancer Cells: An Experimental Review" ***Revista de Oncología*** 4 (2): 77-84, 2002. Article.

8. **Javier A. Menendez**, Inderjit Mehmi, David G. Griggs, and Ruth Lupu. "The Angiogenic Factor CYR61 in Breast Cancer: Molecular pathology and Therapeutic Perspectives" ***Endocrine-Related Cancer*** 10 (2): 141-152, 2003. Review.

9. **Javier A. Menéndez**, Inderjit Mehmi, Ella Atlas, Ramon Colomer, and Ruth Lupu. "Novel Signaling Molecules Implicated in Tumor-associated Fatty Acid Synthase-dependentBreast Cancer Cell Proliferation and Survival: Role of Exogenous Dietary Fatty Acids, p53-$p21^{WAF1/CIP1}$, ERK1/2 MAPK, $p27^{KIP1}$, BRCA1, and NF-κB". ***International Journal of Oncology*** (*in press*) 2003.

10. **Javier A. Menéndez**, Ruth Lupu, and Ramon Colomer. "Pharmacological Inhibition of Fatty Acid Synthase Activity Induces Synergistic Chemosensitization of *HER-2/neu*-overexpressing human breast cancer cells to Docetaxel (Taxotere®)". ***Breast Cancer Research and Treatment*** –Short communication- (*in press*) 2003.

B. Submitted

a. **Javier A. Menendez**, Inderjit Mehmi, Ella Atlas, Bharvi P. Oza, Santiago Ropero, Ramon Colomer and Ruth Lupu. "Pharmacological Inhibition of Fatty Acid Synthase Suppresses Overexpression of the *HER-2/neu* (c-*erb*B-2) Oncogene in Breast and Ovarian Cancer Cells". (*Proceedings of the National Academy of Sciences USA*) 2003.

b. **Javier A. Menendez**, Inderjit Mehmi, Ella Atlas, and Ruth Lupu. "Heregulin Overexpression Protects Human Breast Cancer Cells from Cisplatin-induced Cell Death: Synergistic Sensitization by Trastuzumab in the Absence of *HER-2/neu* Overexpression". (*Cancer Research*) 2003.

c. Ella Atlas, **Javier Abel Menendez**, and Ruth Lupu. "Nuclear transport of Heregulin β2 by a novel nuclear localization signal in its NH_2-terminus" (*Journal of Biological Chemistry*) 2003.

d. **Javier A. Menéndez**, S. Ropero, Ruth Lupu, and R. Colomer. "ω-6 Gamma-Linolenic fatty acid Enhances Docetaxel Cytotoxicity in Human Breast Carcinoma Cells: Relationship to lipid peroxidation and *HER-2/neu* expression" (*Oncology Reports*) 2003.

e. **Javier A. Menéndez**, Inderjit Mehmi, Santiago Ropero, Ella Atlas, Ramon Colomer, and Ruth Lupu. "Breast cancer associated-Fatty Acid Synthase (Oncogenic antigen-519) is insensitive to normal arachidonic fatty acid-induced supression in lipogenic tissues but it is selectively inhibited by tumoricidal alpha-linolenic and gamma-linolenic fatty acids: A novel mechanism by which dietary fatty

acids can alter mammary tumorigenesis" (*Carcinogenesis*). 2003.

f. Javier A. Menendez, Bharvi P. Oza, Ella Atlas, Vishal Verma, Inderjit Mehmi, and Ruth Lupu. "Inhibition of tumor-associated Fatty Acid Synthase antagonizes estradiol- and tamoxifen-induced agonist transactivation of estrogen receptor (ER) in human endometrial adenocarcinoma cells" (*Oncogene*) 2003.

g. Javier A. Menéndez, Inderjit Mehmi, Ella Atlas, and Ruth Lupu. "Heregulin up-regulates breast cancer-associated Fatty Acid Synthase (Oncogenic antigen-519) *via* an autocrine mechanism that requires *HER-2/neu*-dependent activation of MAPK ERK1/2 and phosphatidylinositol 3'-kinase/AKT signaling pathways" (*International Journal of Oncology*). 2003.

h. Javier A. Menéndez, Bharvi P. Oza, Inderjit Mehmi, Ramon Colomer, and Ruth Lupu. "Pharmacological inhibition of breast cancer-associated Fatty Acid Synthase hyperactivity synergistically enhances Taxol-induced cytotoxicity and apoptosis: Involvement of p53, p38 mitogen-activated protein kinase (p38 MAPK) and ERK1/2 MAPK" (*Clinical Cancer Research*) 2003.

i. Javier A. Menendez, Ramon Colomer, and Ruth Lupu. "ω-6 Polyunsaturated Fatty Acid Gamma-linolenic Acid (GLA; 18:3n-6) is a Selective Estrogen Response Modulator (SERM) In Human Breast Cancer Cells: GLA antagonizes Estrogen Receptor (ER)-dependent transcriptional activity, transcriptionally represses ER expression, and synergistically enhances Tamoxifen and ICI 182,780 (Faslodex) efficacy in human breast cancer cells" (*International Journal of Cancer*) 2003.

j. Javier A. Menéndez, Ruth Lupu, and Ramon Colomer. "Inhibition of Tumor-associated Fatty Acid Synthase Activity Enhances Vinorelbine (Navelbine®)-induced Cytotoxicity and Apoptosis against Human Breast Cancer Cells" (*International Journal of Molecular Medicine*) 2003.

C. Manuscripts in preparation

Javier A. Menendez, Inderjit Mehmi, Luciano Vellon, Poh K. Teng, Vishal A. Verma, David Griggs, and Ruth Lupu. "The Angiogenic Factor CCN1 (CYR61) Induces Breast Cancer Cell Resistance to Paclitaxel (Taxol®) through Integrin $\alpha_v\beta_3$-dependent Activation of p42/44 MAPK Signaling Pathway". Article. 2003.

Javier Abel Menendez, Miguel Rubio, Ella Atlas, Judith Campisi, and Ruth Lupu. "Heregulin Induces Telomere Dysfunction in Human Breast Cancer Cells By Regulating TRF-2 Expression: Therapeutic Implications". Manuscript in preparation. 2003.

Javier Abel Menéndez, Santiago Ropero, Sagrario Montero, Hernán Cortés-Funes, and Ramon Colomer. "Synthetic Progestins in Oral Contraceptives Up-Regulate Oncoantigen 519 (Fatty Acid Synthase) in Human Breast Cancer Cells: Relationship to Cell survival and Efficacy of Chemotherapy". Article. 2003.

Santiago Ropero, **Javier Abel Menéndez**, Sagrario Montero, Hernán Cortés-Funes, and Ramon Colomer. "Trastuzumab *plus* Tamoxifen: Growth and Molecular Interactions in Breast Carcinoma" Manuscript in Preparation. 2003.

Javier Abel Menendez, Santiago Ropero, Sagrario Montero, Hernán Cortés-Funes, and Ramon Colomer. "Synergistic Cytotoxicity of Omega-3 Polyunsaturated Fatty Acids (ω-3 PUFAs) and Vinorelbine in Human Breast Cancer Cells: Relationship to ω-3 PUFAs Oxidative Status" Manuscript in Preparation. 2003.

Javier Abel Menéndez, Santiago Ropero, Sagrario Montero, Hernán Cortés-Funes, and Ramon Colomer. "Omega-3 Alpha-linolenic acid Synergistically Enhances Taxanes Cytotoxicity in Human Breast Cancer Cells: Relationship to ω-3 PUFAs Oxidative Status" Manuscript in Preparation. 2003.

E. Abstracts

J. A. Menéndez, M. M. Barbacid, S. Montero, H. Cortés-Funes and Ramón Colomer. "Effect of Oleic acid on the Chemosensitivity of Breast Cancer Cells" **European Journal of Cancer**, 1999; Vol. 35, Supplement 2, p. S14 April. Abstract.
VIII Congress Spanish Association for Cancer Research (ASEICA)
April 19-23, 1999, Sitges, Barcelona, Spain.

M. M. Barbacid, S. Montero, **J. A. Menéndez**, H. Cortés-Funes and R. Colomer. "Kinetic analysis of a Combination of Gemcitabine with Vinorelbine in Human Breast Adenocarcinoma MCF-7 cells *in vitro*". European Journal of Cancer, 1999; Vol. 35, Supplement 2, p. S14 April. Abstract
VIII Congress Spanish Association for Cancer Research (ASEICA)
April 19-23, 1999, Sitges, Barcelona, Spain.

J. A. Menéndez, M. M. Barbacid, S. Montero, E. Escrich, H. Cortés-Funes and R. Colomer. "Taxol and Vinorelbine Cytotoxicity in Human Breast Cancer Cell Lines is Enhanced by Oleic Acid" Proceedings III International Symposium Changes in the Treatment of Breast Cancer, p. 137, June 2-4. Abstract
III International Symposium Changes in the Treatment of Breast Cancer
June 2-4, 1999, Madrid, Spain.

Menéndez, J. A., Ropero, S., Montero, S., Barbacid, M. M., Funes, H-C., and Colomer, R. "Oncogenic Antigen 519 (OA-519) Activity and Expression are Regulated by Fatty Acids in Human Breast Cancer Cells" Proc. Am. Assoc. Cancer Res. Abstract # 1681.
American Association for Cancer Research 92nd Annual Meeting
March 24-28, 2001, New Orleans, USA.

Montero, S., **Menéndez, J. A.**, Ropero, S., Barbacid, M. M., Funes, H-C., and Colomer, R. "Angiogenin Expression is Regulated by Steroid Hormones in Breast Cancer Cells" Proc. Am. Assoc. Cancer Res. Abstract #1275.
American Association for Cancer Research 92nd Annual Meeting
March 24-28, 2001, New Orleans, USA.

R. Colomer, S. Montero, S. Ropero, and **J. A. Menéndez** "Proyectos de Investigación Actuales en el Hospital 12 de Octubre" Encuentros en Oncología, p. 129-132.
Encuentros en Oncología
April 5-6, 2001 Segovia, Spain.

S. Ropero, **J. A. Menéndez**, S. Montero, H-C. Funes, and R. Colomer. "Interactions of Herceptin and Tamoxifen in HER2+ER+ Human Breast Cancer Cells"
IV Madrid Breast Cancer Conference: Changes in the Treatment of Breast Cancer
June 7-9, 2001, Madrid, Spain

J. A. Menéndez, S. Ropero, S. Montero, H-C. Funes, and R. Colomer. "Cerulenin –a Potent Inhibitor of Fatty Acid Synthase- Produces Cytotoxicity Through the p53/p21$^{WAF1/CIP1}$ Pathway and Synergizes with Anti-tubule Agents in Breast Cancer Cells"

IV Madrid Breast Cancer Conference: Changes in the Treatment of Breast Cancer
June 7-9, 2001, Madrid, Spain.

Javier Abel Menendez, Santiago Ropero, Alejandro Vazquez-Martin, Hernan Cortes-Funes, Ruth Lupu, and Ramon Colomer "Trastuzumab and Cerulenin Synergistically Inhibit Breast Cancer Cell Growth: Interaction of HER-2/neu and Fatty Acid Synthase Signaling Pathways" Proc. Am. Assoc. Cancer Res. Abstract #2989.
American Association for Cancer Research 93rd Annual Meeting
April 6-10, 2002, San Francisco, USA.

Alejandro Vazquez-Martin, **Javier Abel Menendez**, Santiago Ropero, Sagrario Montero, Hernan Cortes-Funes, and Ramon Colomer "Anthracycline Resistance on Breast Cancer Cells is Reversed by Inhibiton of Fatty Acid Synthase" Proc. Am. Assoc. Cancer Res. Abstract #4709.
American Association for Cancer Research 93rd Annual Meeting
April 6-10, 2002, San Francisco, USA.

Santiago Ropero, **Javier Abel Menendez**, Sagrario Montero, Alejandro Vazquez, Hernan Cortes-Funes, and Ramon Colomer "Tamoxifen-induced Up-Regulation of HER2 Impairs the Sensitivity to Herceptin in ER+HER2+ Breast Carcinoma Cells" Proc. Am. Assoc. Cancer Res. Abstract #4978.
American Association for Cancer Research 93rd Annual Meeting
April 6-10, 2002, San Francisco, USA.

Javier A. Menendez, I. Mehmi, E. Atlas, M-S. Tsai, and R. Lupu. "Role of Heregulin in Breast Cancer Angiogenesis"
3rd Era of Hope Meeting for the Department of Defense (DOD) Breast Cancer Research Program
September 25-28, 2002, Orlando (Florida), USA.

Javier Abel Menendez, Ramon Colomer, Inderjit Mehmi, Ella Atlas, and Ruth Lupu. "Heregulin/HER2 Signaling Up-Regulates Fatty Acid Synthase (Oncoantigen-519) Expression in Human Breast Cancer Cells Through Activation of the Phosphatidylinositol 3'-Kinase/AKT Kinase Pathway" Abstract #22.
ENH Research Reception
September 19, 2002, Evanston (Illinois), USA.

Javier Abel Menendez, Inderjit Mehmi, Ella Atlas, Miaw-Sheue Tsai, and Ruth Lupu. "The Angiogenic Factor CYR61, a Downstream Effector of Heregulin, Protects Breast Cancer Cells from Paclitaxel-induced Cell Death Through Integrin $\alpha_v\beta_3$" Abstract #366. **European Journal of Cancer** Vol. 38, Supplement 7, p. 108. November 2002.
14th EORTC-NCI-AACR Symposium on "Molecular Targets and Cancer Therapeutics"
November 19-22, 2002, Frankfurt, Germany.

Ruth Lupu, and **Javier A. Menendez**. "Overexpression of the Angiogenic Factor CYR61 Protects Human Breast Cancer Cells from Taxol-induced Cell Death: Involvement of the $\alpha_v\beta_3$/Focal Adhesion Kinase/Phosphatidylinositol 3'-kinase/AKT Kinase Pathway". **Tumor Progression Control and Hormones (non-steroidal) Session** of the **International Congress on Hormonal Steroids and Hormones and Cancer.**
Fukuoka, Japan, October 21-25, 2002.

Javier A. Menendez, Inderjit Mehmi, Ella Atlas, Miaw-Sheue Tsai, David Griggs, and Ruth Lupu. "Overexpression of the Angiogenic Factor CYR61 Protects Human Breast Cancer Cells from Taxol-induced Cell Death: Involvement of the $\alpha_v\beta_3$/Focal Adhesion Kinase/Phosphatidylinositol 3'-kinase/AKT Kinase Pathway" **International Journal of Molecular Medicine.** November 2002.
7th International Meeting on Molecular Oncology

Crete, Greece, 2002.

Javier Abel Menendez, Inderjit Mehmi, Ella Atlas, Ramon Colomer, and Ruth Lupu. "A Molecular Cross-communication Between Heregulin/HER2 and Fatty Acid Synthase in Breast Cancer Cells". Proc. Am. Assoc. Cancer Res. Abstract #6207
American Association for Cancer Research 94th Annual Meeting
April 5-9, 2003. Toronto, Canada.

Javier Abel Menendez, Inderjit Mehmi, Ella Atlas, and Ruth Lupu. "Heregulin Overexpression Protects Human Breast Cancer Cells from Cisplatin-induced Cell Death: Synergistic Sensitization by Herceptin". Proc. Am. Assoc. Cancer Res. Abstract #4053
Selected for presentation in the **Experimental/Molecular Therapeutics Poster Discussion Session** of the **American Association for Cancer Research 94th Annual Meeting**
April 5-9, 2003. Toronto, Canada.

Alejandro Vazquez, Santiago Ropero, **Javier Abel Menendez**, Montserrat Solanas, Eduard Escrich, Ramon Colomer. "Fatty Acid Synthase Regulates Estrogen Receptor in Breast Carcinoma Cells: Relationship to $p21^{WAF-1/CIP-1}$"
Proc. Am. Assoc. Cancer Res. Abstract #2788
American Association for Cancer Research 94th Annual Meeting
April 5-9, 2003. Toronto, Canada.

Ella Atlas, Inderjit Mehmi, **Javier A. Menendez**, Hengameh Zahedkargaran, and Ruth Lupu. "The Immunoglobulin-like Domain of HRGβ-2 is Sufficient for the Chemosensitization and Inhibition of E_2-dependent Colony Formation in MCF-7 cells".
Proc. Am. Assoc. Cancer Res. Abstract #5949
American Association for Cancer Research 94th Annual Meeting
April 5-9, 2003. Toronto, Canada.

Javier Abel Menendez, Inderjit Mehmi, Ella Atlas, and Ruth Lupu. "Heregulin Overexpression Protects Breast Cancer Cells From Cisplatin-induced Cell Death: Involvement of $p21^{WAF1/CIP1}$ and Synergistic Sensitization by Herceptin in the absence of *HER-2/neu* Overexpression." Abstract #12.
ENH Research Reception
March 20, 2003, Evanston (Illinois), USA.

Ruth Lupu, Inderjit Mehmi, Ella Atlas, and **Javier Abel Menendez**. "Overexpression of Heregulin Protects Breast Cancer Cells From Cisplatin-induced Cell Death: Synergistic Sensitization by Trastuzumab in the Absence of *HER-2/neu* Overexpression"
44th Annual Clinical Conference Molecular Therapeutics for Cancer Metastasis. Abstract #19.
March 18-21, 2003. Houston, Texas.

Javier Abel Menendez, Inderjit Mehmi, Ella Atlas, Ramon Colomer, and Ruth Lupu. "Therapeutic Implications of a Novel Bidirectional Molecular Cross-talk Between the *HER-2/neu* and Fatty Acid Synthase Signaling Pathways in Breast Cancer"
44th Annual Clinical Conference Molecular Therapeutics for Cancer Metastasis. Abstract #20.
March 18-21, 2003. Houston, Texas.

Javier A. Menendez, Inderjit Mehmi, David Griggs, and Ruth Lupu. "The angiogenic factor CCN1 (CYR61) protects breast cancer cells from Taxol-induced cell death"
5th Annual Lynn Sage Breast Cancer Symposium Agenda.
September 17-21. Chicago, Illinois.

J. A. Menendez, I. Mehmi, E. Atlas, and R. Lupu. "Overexpression of Heregulin protects breast cancer cells from cisplatin-induced cell death: Synergistic sensitization by trastuzumab in the absence of *HER-2/neu* overexpression"
8th World Congress on Advances in Oncology
and 6th International Symposium on Molecular Medicine
October 16-18, 2003. Crete, Greece.

J. A. Menendez *et al.* "Fatty acid synthase (FAS) inhibitor C75 induces hyperactivation of ERK1/2 MAPK-Estrogen receptor alpha (ER-alpha) cross-talk, loss of ER-alpha expression, inhibition of cell growth, and apoptotic cell death in hormone-dependent breast cancer cells"
2003 AACR-NCI-EORTC International Conference
Molecular Targets and Cancer Therapeutics
Hynes Center, Boston, MA
November 17-21, 2003.

J. A. Menendez *et al.* "Pharmacological blockade of tumor-associated Fatty Acid Synthase (FAS) activity antagonizes estradiol- and tamoxifen-induced agonist transactivation of estrogen receptor-alpha (ER-alpha) in human endometrial adenocarcinoma cells"
2003 AACR-NCI-EORTC International Conference
Molecular Targets and Cancer Therapeutics
Hynes Center, Boston, MA
November 17-21, 2003.